EFFECTS OF PROGESTERONE RECEPTOR MODULATORS ON HUMAN ENDOMETRIUM

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ABSTRACT

The cycling changes of the human endometrium are regulated by the effects of sex steroids such as oestrogen, progesterone (P) and androgens. Molecular studies have elucidated the mechanism of action of sex steroids via sex steroid receptors. This has led to the development of sex steroid receptor ligands, which modulate their function. Progesterone receptor modulators (PRMs) appear promising in their ability to improve common gynaecological symptoms. Only two PRMs are currently licensed for clinical application, mifepristone for medical termination of pregnancy and ulipristal for emergency contraception. However, there is also evidence to demonstrate their potential for the management of benign gynaecological conditions. Asoprisnil is a PRM with partial agonist and antagonist properties and is the most clinically advanced compound in its group. It has been shown to have an endometrial antiproliferative effect in non-human primates and humans and as such to cause reversible amenorrhoea. This effect has been demonstrated in the presence of follicular phase oestradiol (E) levels and is therefore not associated with the side effects of hypo-oestrogenism.

The data presented within this thesis are derived from a phase II multi-centre randomised double-blind placebo-controlled study of asoprisnil administered for 12 weeks to women with symptomatic uterine fibroids scheduled for hysterectomy. Subjects of investigation were clinical effects of asoprisnil on symptoms associated with uterine fibroids. Following hysterectomy, full thickness endometrial tissue samples were obtained for histological assessment, immunohistochemistry and RNA extraction for subsequent quantitative RT-PCR. Effects of asoprisnil on endometrial protein and mRNA expression of proliferation markers, nuclear sex steroid receptors and markers of local immune cell function were subsequently assessed. The extracted RNA was also forwarded for microarray analysis to study the endometrial gene expression profiles after administration of asoprisnil. The aim was to thoroughly evaluate the effects of PRMs, and specifically this novel compound asoprisnil, on endometrium to elucidate its mechanism of action and specify its potential for future clinical applications.

Clinically, asoprisnil showed a remarkable effect on menstrual bleeding pattern with profound suppression and minimal breakthrough bleeding. Quality of life in women with symptomatic uterine fibroids was favourably influenced. Consistent with previous studies, these effects were achieved with maintained serum E levels. A moderate reduction in uterine artery blood flow was demonstrated with asoprisnil indicating that a vascular effect may contribute to its mechanism of action.

The morphology of endometrium following exposure to asoprisnil was thoroughly studied and found to exhibit a unique pattern, inconsistent with previously known histological classifications. Most notable were changes of the glandular appearances as well as the vasculature. Cystic glandular dilatation, previously known to be associated with hyperplasia, was demonstrated without any accompanying hyperplastic features. Specifically, mitotic counts were very low and further studies into proliferation marker expression showed that asoprisnil did not induce endometrial proliferation with a significant inhibitory effect in the stroma. This study lent evidence to the conclusion that administering asoprisnil for 12 weeks does not increase the risk of endometrial hyperplasia or cytological atypia. This was further consolidated by the finding of maintained expression of the tumour suppressor gene PTEN. Distinct vascular changes were found with exposure to asoprisnil, which appeared specific to the endometrium. They consisted of a more frequent occurrence of both aggregates of thin-walled vessels and clusters of vessels with thickened muscularized walls compared to controls. These unique morphological features supported the hypothesis that the effects of asoprisnil are mediated via the endometrial vasculature.

As asoprisnil belongs to the family of sex steroids, its effect on endometrial sex steroid receptor expression was studied on both protein and mRNA level. Sex steroid receptor expression was significantly altered. Notably there was a differential effect in endometrial stroma and epithelium. Specifically, PR was up-regulated in the glands and down-regulated in the stroma. Interestingly, PR was also suppressed in the perivascular cells, which may be significant for the effects of asoprisnil on vascular remodelling. Both ERα and AR expression were up-regulated. The changes in AR expression have previously been cited as a possible mechanism of action for the endometrial antiproliferative effect of asoprisnil.

Microarray analysis was carried out to evaluate endometrial gene expression profiles after administration of asoprisnil compared to controls. There was a notable down-regulation of genes known to be involved in local immune cell function and apoptosis, in particular IL-15. IL-15 was also down-regulated on mRNA level, and as IL-15 is a uterine natural killer (uNK) cell recruiter, the uNK cell marker CD56 was further investigated. CD56 was significantly suppressed by asoprisnil in endometrial stroma and perivascular cells. There is evidence to associate the function of uNK cells with endometrial vascular remodelling. Hence, these effects of asoprisnil on local immune responses may play an important role in the morphological and functional changes in endometrial vasculature and may be highly significant for its mechanism of action.

CONTENTS

DECLARATION

The studies undertaken in this thesis were part of a multi-centre randomised placebo-controlled clinical trial designed and funded by TAP Pharmaceuticals Inc.

The author was responsible for the recruitment and clinical care of the patients from the centre with the most (19) cases (Edinburgh). This involved the performance of screening procedures and all clinical examinations as well as the coordination of clinical visits, study investigations and sample collections and the maintenance of the case report forms. The author was present in the operating theatre at the time of hysterectomy in most cases to ensure appropriate tissue collection.

Immunohistochemical studies and RNA extraction with subsequent Q-RT-PCR were the work of the author except where due acknowledgement is made by reference. Statistical analysis of clinical and laboratory data was carried out at TAP Pharmaceuticals by Mr Cong Han. The gynaecological pathologist Dr Alistair Williams carried out the histological analyses, and the microarray studies including statistical evaluation were done within the Division of Pathway Medicine of the University of Edinburgh under Professor Peter Ghazal, as referred to within the relevant chapters.

No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

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ABBREVIATIONS

ABC	Avidin biotin peroxidase complex
AE	Adverse event
AF	Receptor activation domain
α2M	α2-Macroglobulin
ANCOVA	Analysis of covariance
Ang	Angiopoietin
ANOVA	Analysis of variance
AR	Androgen receptor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary DNA
CL	Corpus luteum
COX	Cyclo-oxygenase
CT	Threshold cycle
DAB	Diaminobenzidine
DBD	DNA binding domain
Deoxy-NTP	Deoxynucleoside triphosphate
dH_2O	Distilled water
Dkk	Dickkopf
DNA	Deoxyribonucleic acid
E	Oestrogen
E_1G	Oestrone glucuronide
ECG	Electrocardiogram
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELA	Evidence of luteal activity
ELISA	Enzyme-linked immunoabsorbent assay
ER	Oestrogen receptor
FAM	6-carboxyfluorescein
FKBP	FK506 binding protein
FRET	Fluorescence resonance energy transfer
Frp	Frizzled related protein
FSH	Follicle stimulating hormone

GNLY	Granulysin
GnRH	Gonadotrophin-releasing hormone
GR	Glucocorticoid receptor
H&E	Haematoxylin and eosin
HMB	Heavy menstrual bleeding
HOX	Homeobox
HPF	High power field
HRE	Hormone response element
HRP	Horse radish peroxidase
HRT	Hormone replacement therapy
HSD	Hydroxysteroid dehydrogenase
HSP	Heat shock protein
IFN	Interferon
IGF	Insulin like growth factor
IGFBP	Insulin like growth factor binding protein
IgG	Immunoglobulin G
Ihh	Indian hedgehog
IL	Interleukin
IUS	Intrauterine system
J867	Asoprisnil
LBD	Ligand binding domain
LNG-IUS	Levonorgestrel releasing intrauterine system (Mirena®)
LSAQ	Leiomyoma Symptom Assessment Questionnaire
$MgCl_2$	Magnesium chloride
MMP	Matrix metalloproteinase
MP	Menstrual pictogram
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
MT-MMP	Membrane type MMP
NBF	Neutral buffered formalin
NCoR	Nuclear receptor co-repressor
NELA	No evidence of luteal activity
NGS	Non-immune goat serum
NHS	Non-immune horse serum
NK	Natural killer
NSAID	Non-steroidal anti-inflammatory drugs
ONA	Onapristone

P	Progesterone
PA	Progesterone antagonist
PBS	Phosphate buffered saline
PBST	PBS + tween 20
PCR	Polymerase chain reaction
PdG	Pregnandiol glucuronide
PG	Prostaglandin
PGDH	Hydroxyprostaglandin dehydrogenase
PH3	Anti-phosphorylated histone H3
PI	Pulsatility index
PLG	Phase lock gel tube
PlGF	Placenta growth factor
PR	Progesterone receptor
PRE	Progesterone response element
PRKO	Progesterone receptor knock out
PRM	Progesterone receptor modulator
PTEN	Phosphate and tensin homologue
Q-RT-PCR	Quantitative reverse transcription polymerase chain reaction
RI	Resistance index
RIN	RNA Integrity number
RMA	Robust Multiarray Average
RNA	Ribonucleic acid
RT	Reverse transcription
RU486	Mifepristone
SAS	Statistical Analysis System
SE	Standard error
SMRT	Silencing mediator of repressed transcription
SPRM	Selective progesterone receptor modulator
SRC	Steroid receptor co-activator
T	Testosterone
TAMRA	6-carboxytetramethyl-rhodamine
TAS	Transabdominal ultrasound
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TVU	Transvaginal ultrasound
UAE	Uterine artery embolization

UFS-QOL	Uterine Fibroid Symptom and Health-Related Quality-of-Life questionnaire
uNK	Uterine natural killer
VEGF	Vascular endothelial growth factor

CHAPTER 1

LITERATURE REVIEW

A more detailed review on relevant subjects may be found within individual chapters

1.1. The Endometrium

1.1.1. The Clinical Perspective

Menstruation is a physiological event. This pivotal reproductive phenomenon may however be associated with a variety of clinically significant symptoms. The severity of symptoms may range from mildly distressing to debilitating, and often there is a disparity between the objective events and the subjective perception of their impact on quality of life. The most common symptoms for which medical advice is sought are heavy menstrual bleeding (HMB) and pelvic pain. Such symptoms may be exacerbated by coexisting uterine or pelvic benign pathology such as fibroids, endometriosis or adenomyosis. Complaints associated with menstruation constitute a significant proportion of the workload in primary care as well as gynaecological specialist clinics. Consultations and subsequent interventions attract considerable cost. Not infrequently do menstrual disturbances indicate surgical intervention, as they are the main presenting symptom for half of the hysterectomies performed in the UK[1]. Further, these complaints appear to be of increasing prevalence. Due to earlier menarche, increased life expectancy, the ability to regulate fertility and less time spent breastfeeding, women nowadays experience more periods than their predecessors a century ago[2]. The average woman in developed countries may expect to menstruate over 400 times during her reproductive life span, and menstrual symptoms are possibly less well tolerated due to more demanding lifestyles of working women[3]. On the other hand, there is a trend to postpone childbearing into later life with the result that management options impairing fertility and certainly hysterectomy are undesirable. Women also increasingly tend to prefer to avoid major surgery when adequately counselled[4]. Hence there is a demand for medical therapies, which are effective and acceptable in the management of symptoms associated with menstruation. In order to develop therapeutic concepts to ameliorate menstrual symptoms, it is essential to have detailed understanding of the mechanisms regulating endometrial events[3].

1.1.2. Endometrial morphology

Human and old-world primate endometrium differs from any other tissue type in its ability to proliferate, transform in preparation for implantation, menstruate in the

absence of pregnancy and then regenerate. Particularly distinctive is its capacity to undergo tissue breakdown at the time of menstruation and subsequent repair[5].

The menstrual cycle may be divided into the proliferative phase, the secretory phase and menstruation in response to the sequential exposure of the endometrium to oestrogen from the developing ovarian follicle, progesterone from the corpus luteum (CL) and progesterone withdrawal with demise of the CL in the absence of pregnancy. Any endometrial section may be morphologically categorized into one of these phases according to the Noyes criteria[6]. Endometrial morphology may be divided into the basal layer adjacent to the myometrium and the uppermost functional layer, where most morphological changes across the cycle are detected. The proliferative phase is characterized by extensive mitoses with proliferation of glandular, stromal and vascular elements. The stroma is compact and the glands narrow and tubular with a thin columnar surface epithelium (Figure 1.1). During the secretory phase, the endometrium differentiates and matures in preparation for implantation. The glands are increasingly tortuous, eventually taking a "saw-toothed" appearance, and the stroma becomes oedematous (Figure 1.2). Finally, in the absence of pregnancy, focal areas of necrosis develop within the functional layer, which is shed at menstruation[7].

Unique to human and old-world primate endometrium are the spiral arterioles, which are crucial to the process of menstruation. These coiled branches of arterial vasculature are present throughout the functional layer of the endometrium (Figure 1.3.) and undergo intense vasoconstriction during the menstrual phase of the cycle. The striking changes in the spiral arterioles were first described by Markee[8], who transplanted endometrium into the anterior chamber of the eye of the rhesus monkey for direct observation during menstruation. These experiments and the findings of arteriolar coiling and constriction at the time the endometrium is shed, constituted the first step towards the understanding of menstrual bleeding. Since then it has been postulated that the perivascular cells, stromal myofibroblasts with contractile activity surrounding the spiral arterioles, are also relevant to the process of menstruation[9].

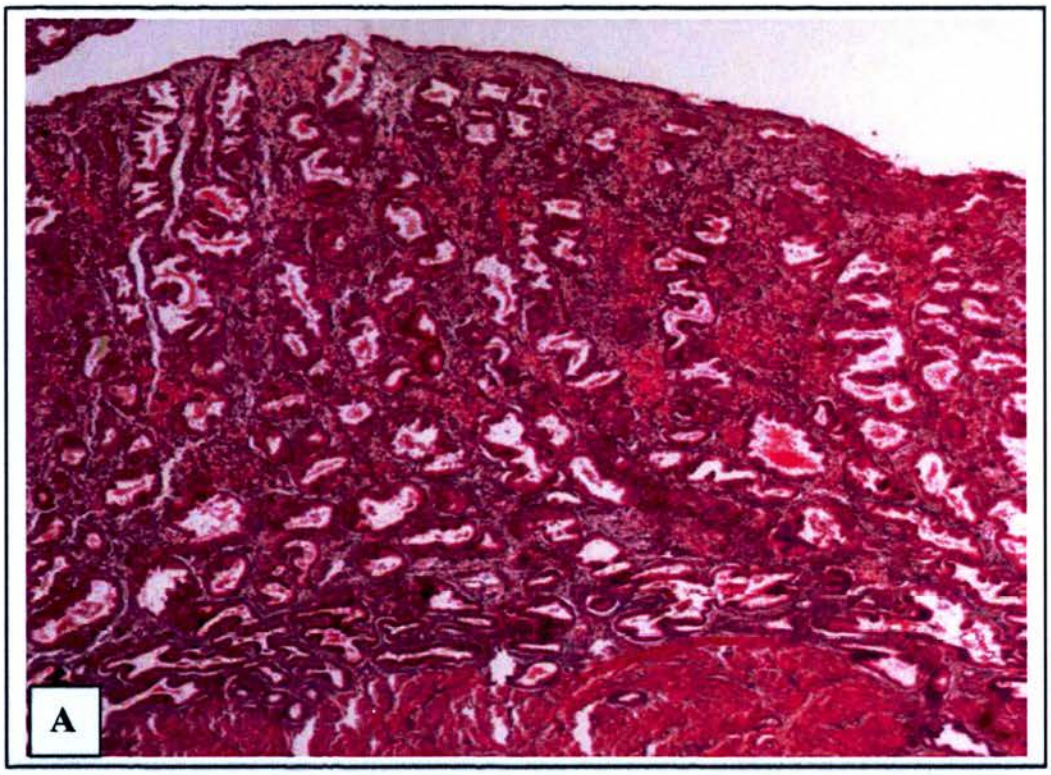
For the understanding of menstrual disturbances, it is equally important to elucidate the mechanisms of postmenstrual endometrial regeneration and repair[5,10]. Re-epithelialization of the vasculature is required, and therefore factors influencing angiogenesis are potentially significant. Based on the experiments by Markee, it was suggested that the observed vasoconstriction led to reduced blood flow and subsequent tissue hypoxia[8].

Figure 1.1. Morphology of proliferative phase endometrium

Endometrium in the proliferative phase of the menstrual cycle with narrow and tubular glands and compact stroma stained with haematoxylin and eosin (H&E); the different layers of the endometrium are highlighted; G: glandular epithelium; S: stroma
(Stained by Miss Morag Hamilton, CRB, Edinburgh;
previously used in MSc thesis by Mrs Teresa A Henderson, University of Edinburgh, 2004)

Figure 1.2. Morphology of secretory phase endometrium

Endometrium in the secretory phase of the menstrual cycle with tortuous glands and oedematous stroma stained with haematoxylin and eosin (H&E) in low power (A) and high power (B)
(Reproduced with permission from Dr Alistair Williams, Department of Pathology, University of Edinburgh)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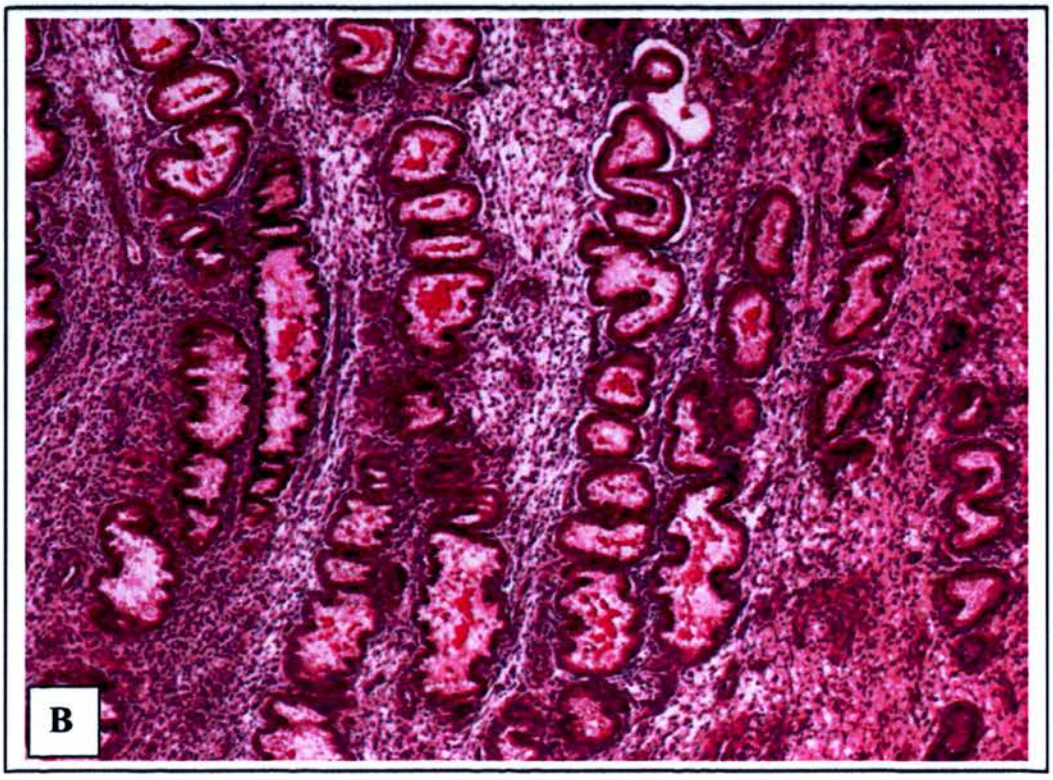
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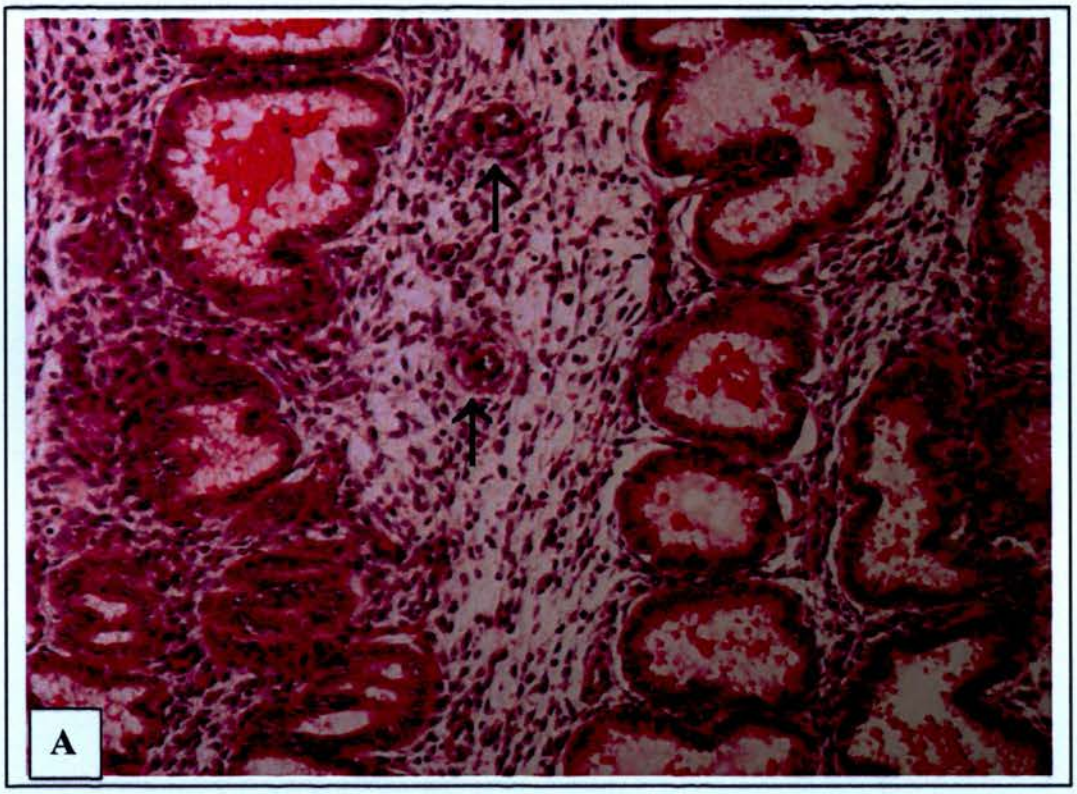
Figure 1.3. Endometrial spiral arterioles

A & B Spiral arterioles are coiled branches of the uterine arterial vasculature, which may be found throughout the functional layer of the endometrium (arrows) (Reproduced with permission from Dr Alistair Williams, Department of Pathology, University of Edinburgh)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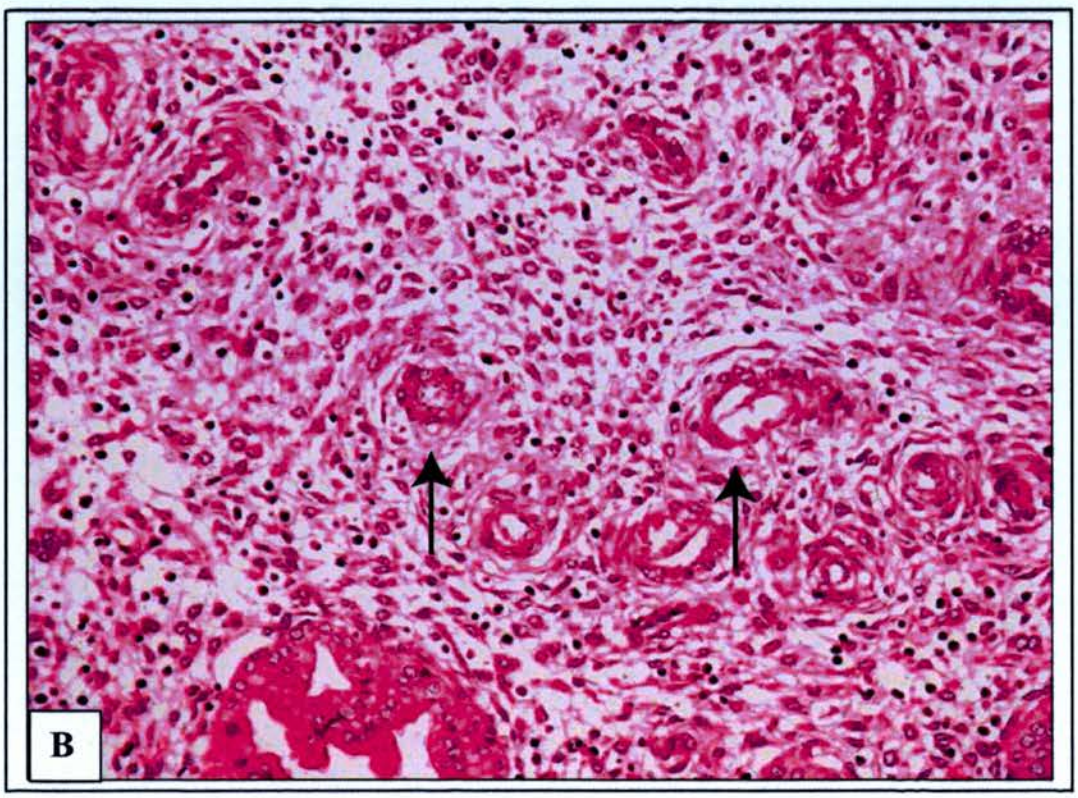
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Since then, a role for hypoxia in the menstrual process via the hypoxia-inducible factor-1alpha has been demonstrated[11] with possible relevance for the onset of menstruation but very likely also for the initiation of subsequent endometrial repair. Hypoxia has been shown to be a strong stimulus for vascular endothelial growth factor (VEGF) expression in endometrial stromal cells[12], and recent studies have indicated VEGF to be essential for endometrial neo-angiogenesis during postmenstrual regeneration[13].

1.1.3. Endometrial proliferation and carcinogenesis

Following tissue injury and repair during menstruation, the endometrium re-enters the proliferative phase under the influence of oestrogen (E). This phase is mainly characterized by proliferation and thickening of the functional layer. Numerous forms of mitosis, which may be directly observed mainly in the early proliferative phase, evidence proliferation. Fewer mitotic cells are observed in the early secretory phase, whilst there is another significant decrease in mitosis in the progesterone (P) dominated mid- and late secretory phases with hardly any detectable cells with mitotic features[14].

The difficult and time-consuming process of determining the mitotic index may be reliably replaced by immunocytochemical assessment. Such assessment across the menstrual cycle has been carried out with anti-phosphorylated histone H3 (phospho H3) and found to be highly correlated with the mitotic cell count. Analysis of variation in mitotic index and immunocytochemistry with both phospho H3 and the less specific proliferation marker Ki-67 have all indicated that most proliferation occurs during the proliferative phase with negligible evidence in the mid- and late secretory phases[14] (Figure 1.4.).

Endometrial proliferation is essential for the maintenance of the menstrual cycle and to prepare for implantation. As such, it is a physiological event. There is a potential for persistent proliferation to initiate endometrial pathology, when it leads to hyperplasia, particularly when complex and associated with cellular atypia. Morphological changes and an increased mitotic index are indicators of an increased risk of malignant transformation. Even prior to any histological changes, the down-regulation of the tumour suppressor gene phosphatase and tensin homologue (PTEN) has been described as an early event in endometrial carcinogenesis and can be detected by immunohistochemistry[15-17]. PTEN has been suggested as a biomarker for premalignant endometrial disease, as it appears to serve as a gatekeeper for the initiation of carcinogenesis.

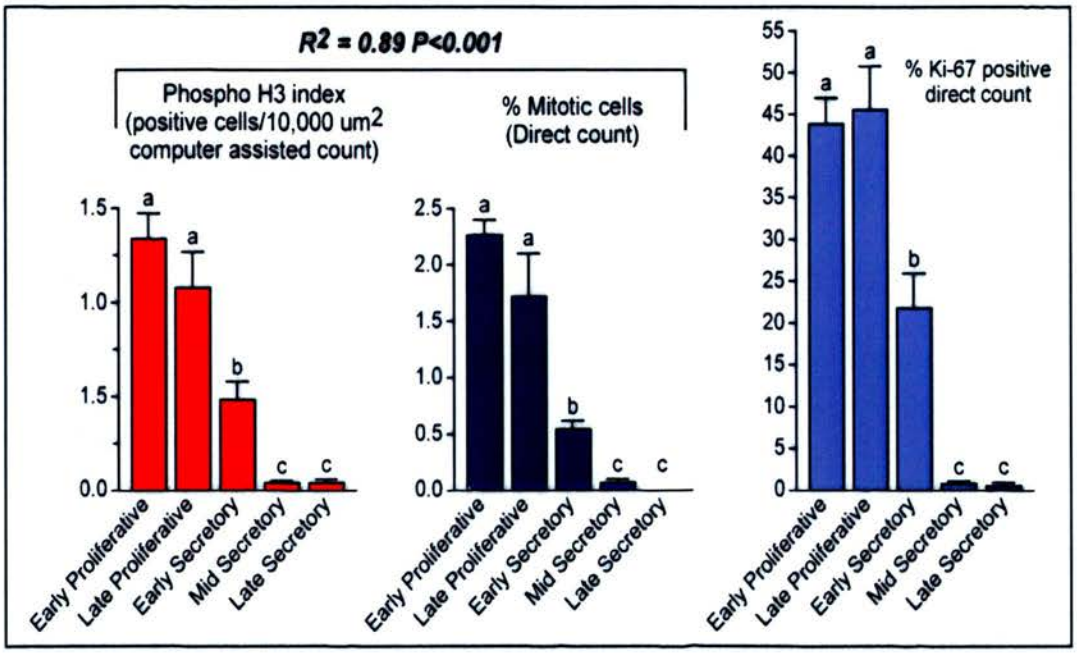


Figure 1.4. Mitosis and proliferation marker expression across the menstrual cycle

Comparison of three proliferative indices in human endometrium during the menstrual cycle

Both direct count of mitosis and proliferation marker expression indicate a pattern of elevated mitotic activity in the proliferative phase, which significantly decreases in the secretory phase;

there is a high correlation between the Phospho H3 index and direct count of mitotic figures ($R^2 = 0.89$, $P < 0.001$) and also between the mitotic index and Ki-67 expression ($R^2 = 0.74$, $P < 0.05$)

(Derived with permission from:

Brenner et al., Human Reproduction 2003; 18: 1185-93)

The most common form of endometrial cancer, endometrioid endometrial adenocarcinoma, shows inactivation of PTEN in up to 83% with frequent mutations and deletions[15]. Endometrioid endometrial cancer has also been associated with the risk factor of exposure to unopposed E. P has been shown to play an important role in eliminating PTEN-deficient endometrial cells[18], indicating the dependency of endometrial physiological and pathological events on sex steroid hormones.

1.1.4. Regulation of endometrial events by sex steroid hormones

It has been firmly established that the endometrial changes through the menstrual cycle are regulated by the sequential exposure to the ovarian steroids oestradiol (E) and progesterone (P)[19]. The level of E rises during the proliferative phase, when the ovarian follicle matures, whilst P depends on ovulation having occurred, as it is produced by the CL, and therefore peaks in the secretory phase. E is essential for endometrial proliferation, and secretory transformation with stromal decidualization in preparation for implantation is dependent on P. Menstruation is the endometrial response to withdrawal of P due to the demise of the CL in the absence of pregnancy. Concerted ovarian production and release of both E and P is essential to induce consequent changes within the endometrium and a regular cycle pattern. Other sex steroids such as androgens and glucocorticosteroids have also been shown to have a modulating effect[20].

The action of sex steroid hormones is mediated via their respective receptors. Relevant for endometrial function are the progesterone receptor (PR), oestrogen receptor (ER) with its subtypes ERα and ERβ, androgen receptor (AR) and the glucocorticoid receptor (GR). Endometrial expression of these receptors is temporally and spatially regulated in the respective endometrial tissue compartments. It is particularly variable in the glandular epithelium and most pronounced in the functional layer. These variations occur in relation to the menstrual cycle phases suggesting a feedback mechanism between sex steroid hormones and their receptors. PR expression is under dual control of E and P with up-regulation in the proliferative phase and subsequent down-regulation by its own ligand[21]. As PR synthesis is dependent on ligand interaction with the ER, the presence of PR indicates a functional ER-mediated pathway[20]. Similarly, ER expression increases throughout the proliferative and until the early secretory phase with a subsequent decline in the mid- and late secretory phases. Both GR[22,23] and AR[24] are predominantly expressed in endometrial stroma with AR gradually decreasing from the early proliferative through to the late secretory phase[24]. Steroid receptor co-activators and co-repressors

are also likely to play an important role in regulating endometrial function. Such roles however have as yet not been fully determined[25-27].

Crucial to the process of menstruation is the regulation of the endometrial vasculature, and whilst the straight arteries in the basal layer are not influenced by hormonal changes, the spiral arterioles are steroid responsive. In this context, it is notable that stromal PR is specifically localized in the perivascular regions[19]. The findings that perivascular but not endothelial cells express PR[28], indicate that the perivascular cells may act as a mediator for the effect of P on the vasculature. Endothelial cells have been shown to express ERβ and GR. It has been demonstrated in the rhesus macaques as well as the human[29,30] that the ER subtype expressed in endothelial cells is ERβ, whilst ERα, PR and AR are all absent. Isolated and cultured human endometrial endothelial cells have been found to express ERβ mRNA but not ERα, and interestingly also PR mRNA[31]. Up-regulation of stromal and vascular ERβ expression has been shown in the late secretory phase, with concurrent down-regulation in the glandular epithelium. The stromal cells expressing ERβ in this phase of the cycle were observed mostly around spiral arterioles[32]. The hypothesis for the modulation of endometrial vasculature by sex steroids is therefore either a direct effect via the endothelial ERβ or an indirect effect through paracrine signalling from the perivascular cells or both[30]. Interestingly, the endothelial cells express GR[22], which is also present in the endometrial stroma, fibroblasts, lymphocytes and uterine-specific natural killer (uNK) cells[23] but absent from the glandular epithelium. The exact significance of these sex steroid receptor expression patterns for vascular function has not been fully elucidated.

1.1.5. The role of the local endometrial immune system

Menstruation is triggered by a change in sex steroid levels, in particular P withdrawal. However, sex steroids are not the only regulators of the menstrual process, and the immune system has been attributed an important role[33,34]. P withdrawal initiates a cascade of events leading to the influx of leukocytes into the endometrium via the expression of cytokines. This leukocytic invasion and subsequent production of inflammatory mediators has led to the description of menstruation as an inflammatory event[35]. The menstrual process may be divided into two phases[36]. The early events of vasoconstriction of the spiral arterioles and cytokine changes are hormone dependent and reversible by adding back P within 36 hours. The subsequent activation of lytic mechanisms, probably as a consequence of hypoxia, is inevitable and independent of the sex steroid environment. Activation of

matrix metalloproteinases (MMPs) such as MMP-3 with the potential to degrade the extracellular matrix (ECM) can lead to a whole cascade of subsequent lytic enzyme activation and eventually to tissue sloughing and menstrual bleeding[10].

Particular significance for this process appears to be attributable to the perivascular cell, which is in close relation to the endothelial cells and, unlike those, expresses sex steroid receptors. The perivascular cell is responsive to changes in P levels and also expresses chemokines, cytokines and prostaglandins (PG), all important for the control of leukocyte entry into the endometrium. It has also been shown to express CD40, activation of which up-regulates interleukin-8 (IL-8) and the enzyme responsible for PG synthesis cyclo-oxygenase 2 (COX-2)[9]. Indeed, an increase of key inflammatory mediators IL-8 and COX-2 has been demonstrated at both mRNA and protein level 48 hours following P withdrawal[37]. The properties of the perivascular cell are very likely to facilitate the invasion of leukocytes in the immediate premenstrual phase.

A significant population of leukocytes may be found in the endometrium including neutrophils, lymphocytes, macrophages, mast cells and other haematopoetic cells. Whilst their proportion remains stable in the basal layer, there is considerable fluctuation in the functional layer from around 8% bone marrow derived cells in the stroma in the proliferative phase to at least 20% in the late secretory phase[38]. There is also a variation of leukocyte distribution across the cycle with scattered occurrence throughout the stroma in the proliferative phase and small aggregates around glands and spiral arterioles in the late secretory phase. These leukocytes are further sources of cytokines, which potentially induce a spiralling inflammatory response[9]. Some leukocytes also release MMPs and MMP activators initiating the breakdown of the ECM[33,39].

The majority of the leukocyte population in the late secretory phase consists of uterine natural killer (uNK) cells. The proportion of this cell type rises to 70% in early pregnancy[40]. These cells have a unique phenotype ($CD56^{bright}$, $CD16^{-}$, $CD3^{-}$) and are thus distinguishable from peripheral blood NK cells ($CD56^{dim}$, $CD16^{bright}$, $CD3^{-}$). It is yet to be established whether the dramatic increase in uNK cells during the mid-late secretory phase is due to in situ proliferation or de novo migration from the peripheral circulation. In any case, the appearance of uNK cells does vary according to the menstrual cycle suggesting hormonal regulation, even though this cell type does not express ERα or PR[23,41]. Once again, the perivascular cells appear to have a paracrine role, as they have been shown to release IL-15[42,43], which stimulates proliferation of CD56+ve uNK cells. A clear link has been demonstrated between progesterone levels, IL-15 expression and fluctuation of uNK cells in the

endometrial stroma[43]. Interestingly, whilst immunonegative for ERα and PR, uNK cells have been shown to express ERβ1 and GR, raising the possibility that oestrogens and glucocorticoids may have a directly modulating effect on these cells via those steroid receptors[23]. The exact role of uNK cells is incompletely understood but appears to be important for decidualization, implantation and menstruation[44]. Several observations have pointed to the association of uNK cells with the process of decidualization. There is also some evidence for the significance of the uNK cell for the initiation of menstruation. Even prior to the morphological signs of impending menstrual tissue breakdown such as infiltration of neutrophils, stromal cell clumping and interstitial haemorrhage, there is an apparent apoptosis of uNK cells[40] coinciding with the fall in P levels. Some uNK cells have been shown to produce an enzyme that can activate latent MMP-2 (MT1-MMP) and hence lead to tissue degradation[45]. uNK cells have also been demonstrated to produce angiogenic growth factors (i.e. vascular endothelial growth factor C (VEGF-C), placenta growth factor (PlGF) and angiopoietin 2 (Ang-2)) and might therefore contribute to the process of postmenstrual endometrial regeneration[46].

Further studies are clearly required to fully elucidate the mechanisms responsible for remodelling the endometrium. So far, many studies have examined the regulators of the endometrial tissue breakdown at the time of menstruation. Far less is known about the subsequent tissue repair and its modulating factors, and yet the repair mechanisms are likely to be of equal relevance to the full understanding of normal and abnormal uterine bleeding[5,10]. Currently, there is limited insight into the reasons why in some women menstruation is associated with severely distressing symptoms such as heavy bleeding and / or dysmenorrhoea in the absence of any pathology.

1.2. The Progesterone Receptor and its Ligands

1.2.1. The Progesterone receptor (PR)

1.2.1.1. Steroid receptor biology

The PR is an intranuclear receptor belonging to a steroid-thyroid-retinoid receptor superfamily, which includes receptors for steroids, thyroid hormone, vitamin A and D derived hormones and certain fatty acids[47]. The steroid receptor subgroup (Type I receptors) comprises the PR, ER, AR, GR and the receptor for mineralocorticoids (MR)[20,48]. Members of the nuclear receptor superfamily share a common structure with five functional domains, denoted A/B, C, D, E and F[49] (Figure 1.5.).

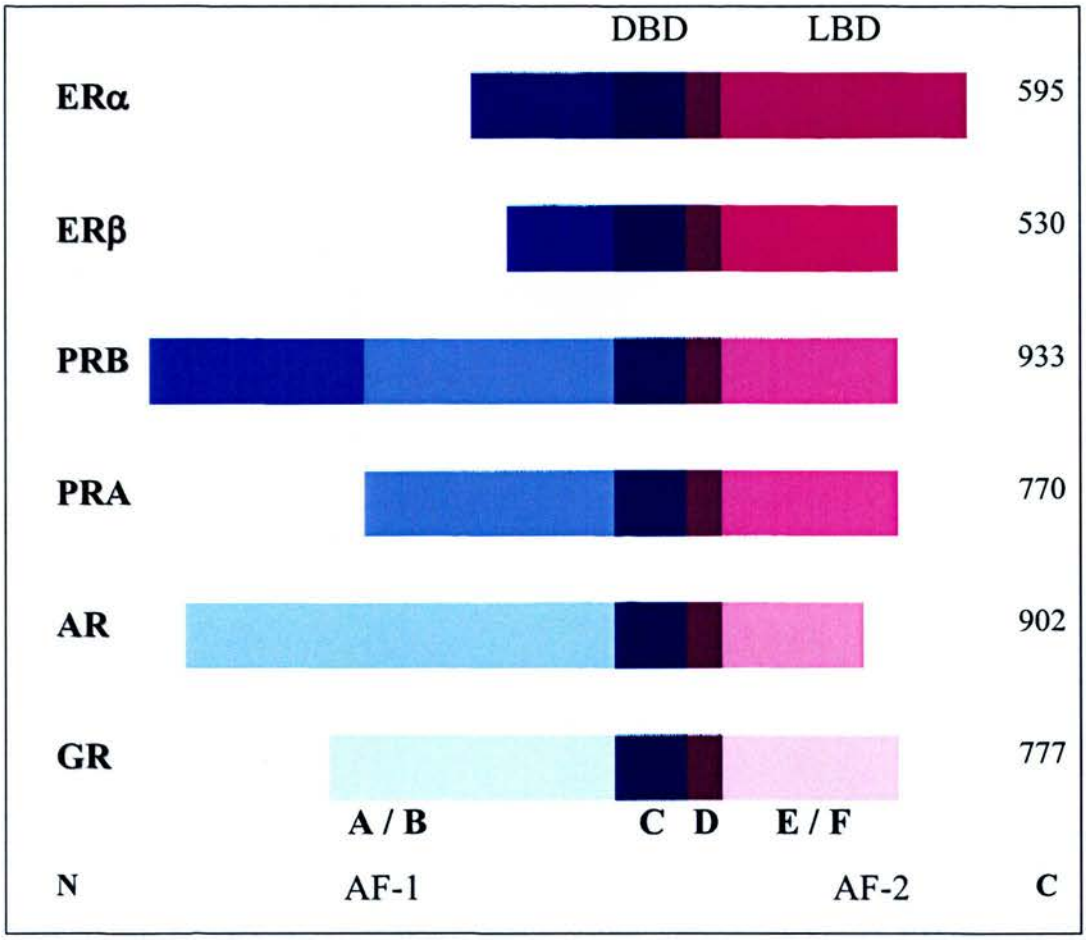


Figure 1.5. The sex steroid receptor superfamily

Five functional domains (A/B, C, D, E and F) are common to the structure of all nuclear receptors;
the DNA-binding domain (DBD) is contained in the C domain and the ligand-binding domain (LBD) is located in the E domain; A/B is the N-terminal end (N) and F is the C-terminal end (C); D is the hinge region; AF indicates activation function
(Adapted from:
Critchley & Saunders, Reproductive Sciences 2009; 16 (2): 191-199)

The C domain contains a highly conserved DNA-binding domain (DBD) determining the specificity of the receptor for particular hormone responsive elements (HREs). It also enables interaction between the receptor and its target genes. The E domain is the ligand-binding domain (LBD), which determines whether the receptor is activated. In the absence of specific ligands, the PR is maintained in an inactive state in the cytoplasm by association with a large complex of chaperone proteins such as heat shock proteins (HSP) and FK506-binding proteins (FKBPs), which can be found in complex with several steroid receptors[49,50]. The interaction between the PR and the chaperone complex is critical in order to maintain the functionality of the receptor and the competence to bind P. FKBP4, bound to the inactive PR via the adapter protein HSP90, has been demonstrated to potentate PR transcriptional activity, whilst its absence results in reduced uterine receptivity[50]. Ligand binding renders the PR transcriptionally active. Alternatively, there is a ligand-independent mechanism whereby an increase in intracellular kinase activity and stimulation of phosphorylation pathways can lead to PR activation[51]. Once activated through either ligand-dependent or independent mechanisms, the phosphorylated PR translocates to the nucleus and binds to a progesterone responsive element (PRE) on target DNA to alter gene expression with a promoting or repressive effect. Additionally, transcription activation is mediated via recruitment of co-regulators (co-activators or co-repressors) with an enhancing or inhibiting effect[27]. Co-activators such as acetyltransferase proteins and steroid receptor co-activator members (SRC1, SRC2, SRC3) accelerate the formation or increase the stability of the pre-initiation complex for activation of transcription. On the other hand, the non-ligand bound PR can suppress basal transcription through co-repressors such as nuclear receptor co-repressor (N-CoR) and silencing mediator of repressed transcription (SMRT). However, the PR has been shown to regulate gene expression not just directly or via co-regulators but also through crosstalk with other transcription factors and by activating kinase signalling cascades from the cytoplasm[50]. The target genes of regulation by PR on a molecular level have only partially been identified so far[52]. Indian hedgehog (Ihh) has been described as a target gene induced by P via the PR. Studies in mouse models have shown Ihh to be expressed in the glandular and surface epithelium with the downstream targets of the Hedgehog signalling pathway located in the stroma[53]. It has been suggested that Ihh may be involved in coordinating communication between the epithelial and stromal tissue compartments[50].

1.2.1.2. The function of PR and its isoforms

The PR is an essential coordinator for the regulation of female reproductive activities by P, including endometrial receptivity, implantation and maintenance of pregnancy, mammary gland development and sexual behaviour. Evidence for the role of PR in reproductive function has been derived from mouse models, in which the PR has been ablated (PRKO)[51]. Ablation of PR results in pleiotropic reproductive abnormalities. Experiments with PRKO mice have provided proof that PR is an essential mediator of ovulation in the mouse. PR is specifically required for the follicular rupture, whilst luteinization of the granulosa cells can still occur in the PRKO mouse. The effects of P on the endometrium mediated by PR include the inhibition of E induced epithelial hyperplasia. On the other hand, the stromal effects of P are proliferation of the spiral arterioles[54] and differentiation. PRKO mice are found to have a markedly hypocellular endometrial stroma, whilst the epithelium is hyperplastic due to unopposed action of E. The potent anti-inflammatory response mediated by PR is abolished in these animals, and the stroma is infiltrated by neutrophils and macrophages. Proliferation and differentiation of the developing mammary gland has also been demonstrated to depend on the effects of P and PR[51].

Several isoforms of the PR have been identified, including PR-A, PR-B, PR-C and PR-M[19]. The two main isoforms described in the human are PR-A (Mr 94,000) and PR-B (Mr 120,000). They arise from a single gene with specific promoters for the two different subtypes[55]. PR-A is the shorter isoform and is devoid of 164 amino acids at the N-terminus, which are present in PR-B. Otherwise the two subtypes are identical with indistinguishable hormone- and DNA-binding affinities. Both PR-A and PR-B are co-expressed in endometrial target cells[56]. Depending on the relative ratio, they can dimerize into homodimers (A:A or B:B) or heterodimers (A:B). Both PR-A and PR-B and the dimers are capable of binding P and interacting with PREs, but they are functionally different[51].

Selective ablation of either subtype in mice has provided models (PRAKO and PRBKO) to study their differential function[49,51,57,58]. PR-A appears to be less transcriptionally active than PR-B. Ovarian and uterine functions remain unaffected in the PRBKO mouse indicating a critical role for PR-A in mediating the ovarian response to P. Ovulation is severely impaired in the PRAKO mouse. PR-A is essential for decidualization and for the anti-inflammatory effect of P on the endometrium. PRAKO mice have an absent decidual response and are infertile. Regulation of endometrial growth also depends on PR-A, as in the PRAKO mouse E induces endometrial hyperplasia, which cannot be suppressed by P. The function of PR-B appears sufficient to elicit a normal proliferative and differentiative response of

the mammary gland to P. There is no appreciable effect in the PRAKO mouse on mammary gland function, whilst this is severely impaired in the PRBKO mouse, particularly the development in pregnancy[49,59].

In addition to these findings that the two receptor subtypes appear to regulate different target genes, PR-A has been suggested as an inhibitor of PR-B activity[60] with an effect of overall diminished P responsiveness. This repressive capability of PR-A also appears to extend to E, glucocorticoid and mineralocorticoid receptor-dependent transcriptional activation. It appears that the respective cellular response to P depends on the differential expression of the receptor subtypes, and the ratio of PR-A and PR-B expression is important[51]. Notably, there is also a difference in response of the two PR isoforms to PAs. Whilst the binding of a PA inactivates PR-A, the effect on PR-B can result in conversion to an active transcription factor with modulation of intracellular phosphorylation pathways[51].

1.2.1.3. Spatio-temporal distribution of PR in the endometrium

The variation of PR expression in the endometrium across the menstrual cycle has been well described[19,56,61,62]. Expression in the functional layer, which is shed at menstruation, and in the basal layer of the endometrium is differentially regulated. In the basal layer, PR persists both in glands and in stroma throughout the menstrual cycle. In the functional layer, PR is predominantly expressed in the proliferative phase. In the epithelium, it is then down-regulated by its own ligand P following ovulation[21]. PR persists in the stroma, particularly in perivascular location surrounding the spiral arterioles, in a distribution similar to pregnancy[29,63]. The ratio of PR-A to PR-B expression increases in the secretory phase indicating that P preferentially down-regulates PR-B in both stroma and glands[56,64]. This is further evidence for PR-A being the mediator of P-regulated events in the secretory phase such as decidualisation[65].

1.2.2. PR ligand - Progesterone (P)

1.2.2.1. The hormone

P (pregn-4-ene-3, 20-dione) is a C-21 steroid hormone ($C_{21}H_{30}O_2$), first discovered in the mid 1930s[66,67] (Figure 1.6.). P is the main naturally occurring progestogen, whilst progestins are synthetic progestogens. P is predominantly produced in the ovaries with peak levels following ovulation in the secretory phase of the menstrual cycle, as its main source is the corpus luteum. It is also produced in the brain and in significant amounts in the placenta after the 8th week of gestation[68].

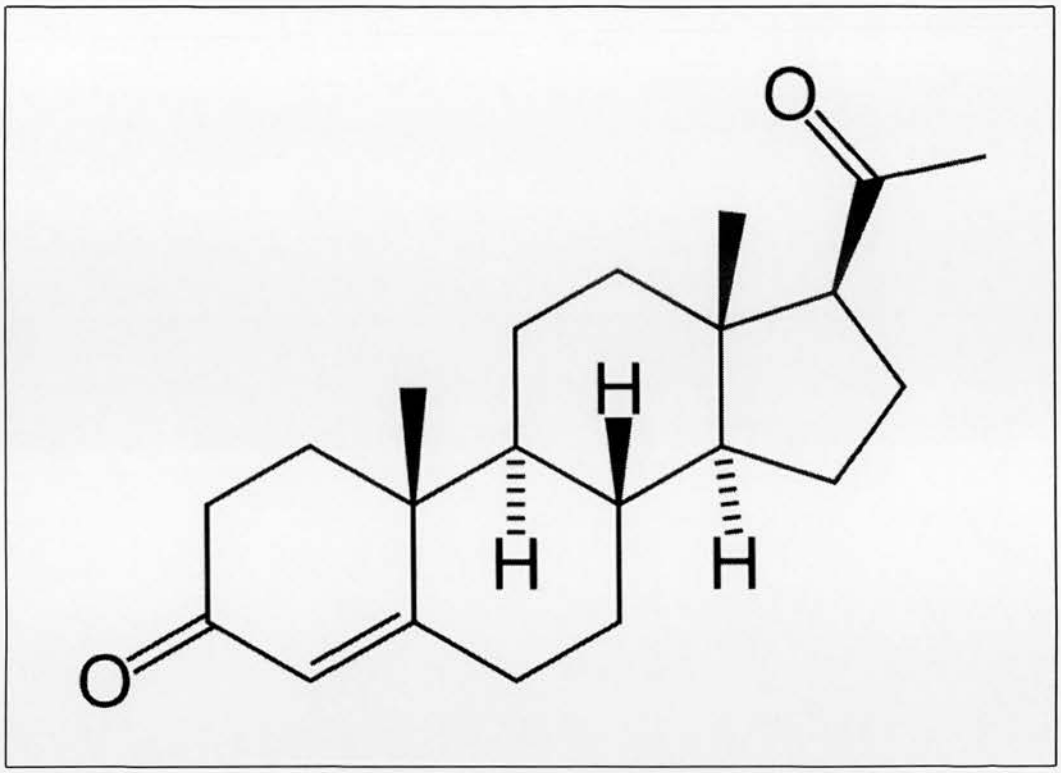


Figure 1.6. Chemical structure of progesterone

Pregn-4-ene-3, 20-dione is a C-21 steroid hormone ($C_{21}H_{30}O_2$)

Levels are low in children and postmenopausal women and vary according to the menstrual cycle phase during the reproductive years. A serum level of >30 nmol/l on day 21 of a 28-day cycle is indicative of ovulation having occurred. High levels are reached in pregnancy and may rise up to 600 nmol/l at term[69].

The precursor of all steroid hormones is cholesterol, which is then oxidized to pregnenolone (Figure 1.7.). Pregnenolone is converted into P in two steps of oxidization followed by a keto/enol tautomerization reaction. P itself is a precursor of other steroid hormones, such as mineralocorticoids and, after conversion into 17-hydroxyprogesterone, cortisol and androstenedione. Androgens and oestrogens can be derived from androstenedione. Total synthesis of P was first described in the 1970s[70]. P is also found in certain plants, particularly the yam family Dioscorea, and synthesized by yeast[71]. P is metabolised in the liver and then excreted by the kidneys with a half-life of about 25-50 hours.

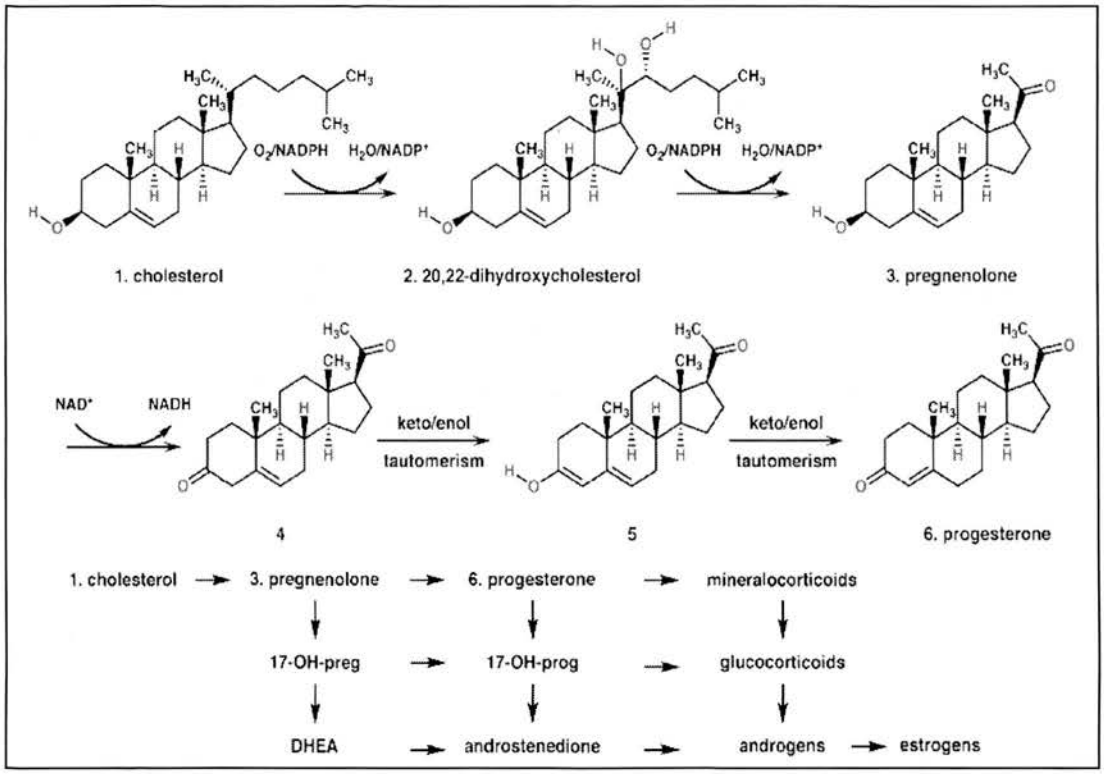


Figure 1.7. Synthesis of progesterone from cholesterol

Cholesterol is the precursor of all steroid hormones; it is oxidized to pregnenolone and then converted into progesterone by oxidation and a keto/enol tautomerization; other steroid hormones are derived from progesterone such as mineralocorticoids, cortisol, androstenedione and subsequently androgens and oestrogens
(Derived from:
http://en.wikipedia.org/wiki/File:Progesterone_biosynthesis.png#file)

1.2.2.2. Progesterone function

P is a key regulator of female reproductive activity, and its effects are mainly mediated via the PR as described above[72]. Whilst the reproductive system is its main target, P also affects other functions such as myelination and neuroprotection[73], regulation of immune responses, regulation of core temperature and relaxation of smooth muscle, particularly in the bronchi. Within the uterus, P predominantly contributes to the regulation of endometrial function. P is essential for the transformation of the endometrium into a state of receptivity in preparation for implantation[74,75]. Animal studies have investigated the expression of P-regulated genes, which may be significant for the successful establishment of a pregnancy and hence for the management of infertility[76-78]. Endometrial decidualization is P-dependent as are the maintenance of pregnancy and the process of menstruation. The effect of P relies on endometrial priming by E, particularly as E mediates up-regulation of the PR. The endometrium is most susceptible to a P effect in the mid secretory phase, which represents both the implantation window and a period of increased sensitivity to P withdrawal and hence is critical to both events of implantation and menstruation[19].

At the molecular level, markers of P function and endometrial genes regulated by P have been described, such as insulin-like growth factor binding protein (IGFBP)-1, prolactin, glycodelin, 15-hydroxyprostaglandin dehydrogenase (PGDH) and calcitonin. More recently, Dickkopf-1 (Dkk-1) mRNA synthesis and protein expression has been reported to be regulated by P in human endometrial stromal cells[79]. Dkk-1 is an inhibitor of the canonical Wnt signalling pathway and may thus influence endometrial differentiation. A dramatic up-regulation of Dkk-1 in the endometrial stroma has been demonstrated in the secretory phase of the menstrual cycle. At the same time, frizzled related protein (Frp) HE, another inhibitor of the Wnt signalling pathway, is down-regulated. A role for Wnt signalling in the dialog between epithelial and stromal compartments has been suggested, and the temporally differential expression of FrpHE and Dkk-1 highlights the importance of P in the regulation of this pathway[80]. Dkk-1 is also involved in control of the homeobox (HOX) genes. HOX genes are essential regulators of development[81], and particularly HOXA10 plays an important role in implantation[82] but also in the development of endometriosis and polycystic ovarian disease[19]. Expression of HOXA10 has also been shown to be regulated by P in a differential tissue-specific manner. Whilst P induces HOXA10 in the endometrial stroma, the effect of P on HOXA10 in the myometrium is down-regulation[83].

The implications of P in the modulation of physiological functions but also the development of pathological conditions have been recognized. Whilst traditionally, the growth of uterine leiomyomata was thought to be E dependent, the contributory role of P has now also been appreciated[84]. There is both in vitro[85] and clinical evidence[86] that P as well as E is an important promoter of leiomyoma growth.

1.2.2.3. Progesterone applications

With increasing insight into the mechanism of action of sex steroids, P and progestins have been used in therapeutic approaches to modulate reproductive function, to ameliorate associated symptoms such as heavy menstrual bleeding (HMB) and dysmenorrhoea and to manage other gynaecological conditions. There is a wide range of applications for progestogens, and they may be administered by various routes. Progestogens have a major role in contraception, assisted reproductive technology and hormone replacement therapy. They have a place in the treatment of endometriosis, polycystic ovarian disease and also endometrial hyperplasia. Progestogens have been used to control menstrual disturbances and are particularly effective if these are associated with anovulatory cycles[87,88]. The route of administration is important, as any compound will be metabolised passing through intestine and / or liver, and hence the respective tissue effect will be dependent on the hormonal activity of the metabolites in the circulation. Additionally, it will be determined by intracellular mechanisms, which can be used to an advantage, when tissue-specific steroid metabolising enzymes are regulated by synthetic compounds[89]. Currently, an efficient way of administering exogenous progestogens to the endometrium is via the levonorgestrel releasing intrauterine system (LNG-IUS). This route achieves maximal levels of the androgenic progestogen levonorgestrel within the endometrium with relatively low systemic levels[90]. The LNG-IUS induces extensive decidualization and endometrial atrophy with a profound effect on bleeding pattern[91]. The most frequent indication for discontinuation of treatment with progestogens has been unscheduled breakthrough bleeding, which has been attributed to modifications of the endometrial vasculature with increased vessel fragility and changes in hemostasis[19,92-94]. A marked down-regulation of ER and PR has been described in the initial response to the insertion of a LNG-IUS. 17β-hydroxysteroid dehydrogenase (HSD)-2 remains elevated with the effect of continued conversion of oestradiol to the less potent oestrone. It has been postulated that the resulting local intracellular E-deficient environment may contribute to increased vascular fragility[93,95]. During the first six months after insertion of the device, an enhanced expression of IL-8 and COX-2 has been demonstrated leading to

higher local concentrations of prostaglandins. Altered expression of local inflammatory mediators may affect vascular integrity and hence predispose to the side effect of unscheduled bleeding episodes[91].

1.2.3. PR ligands

The discovery of the PR[96-98] opened up the potential for the development of synthetic PR ligands. The first description of mifepristone (RU 486), a PR and glucocorticoid receptor antagonist, was published in 1981[99]. This was followed by reports on its effect on menstruation[100] highlighting the potential for cycle regulation and birth control[101]. Subsequently, numerous compounds have been developed with a varied spectrum of PR agonistic and antagonistic properties. This large group of synthetic PR ligands ranges from pure agonists such as progestins to pure antagonists (Figure 1.8.). The compounds exhibiting partial and mixed agonist/antagonist activity have been classified as selective progesterone receptor modulators (SPRMs) and will be described further in the next section.

1.2.3.1. Mifepristone (RU 486)

Mifepristone (RU 38486) [11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-propinyl-4,9-estradiene-3-one] is a beta-aryl-substituted, 19-nortestosterone-derived compound (Figure 1.9.). It strongly antagonizes all effects of P including the endometrial preparation for implantation and maintenance of pregnancy. Hence, not surprisingly, its first clinical application was termination of pregnancy[102].

Unfortunately, this has subsequently delayed and obstructed further development of mifepristone and other compounds with P antagonistic properties for alternative clinical use, as large pharmaceutical companies have distanced themselves from the negative image of a drug that is capable of inducing abortion. Nevertheless, mifepristone and other PAs have many other potential indications in gynaecological and non-gynaecological practice, some of which have been described and tried previously[103].

Long-term administration of mifepristone in benign gynaecology has particularly been described in the context of contraception[104] and management of menstrual disturbances, uterine fibroids and endometriosis. Administration of low-dose mifepristone for 3-6 months has been reported to result in significant suppression of menstrual bleeding, regression of uterine fibroid volumes[105] and improvement in endometriosis-related symptoms[103,106].

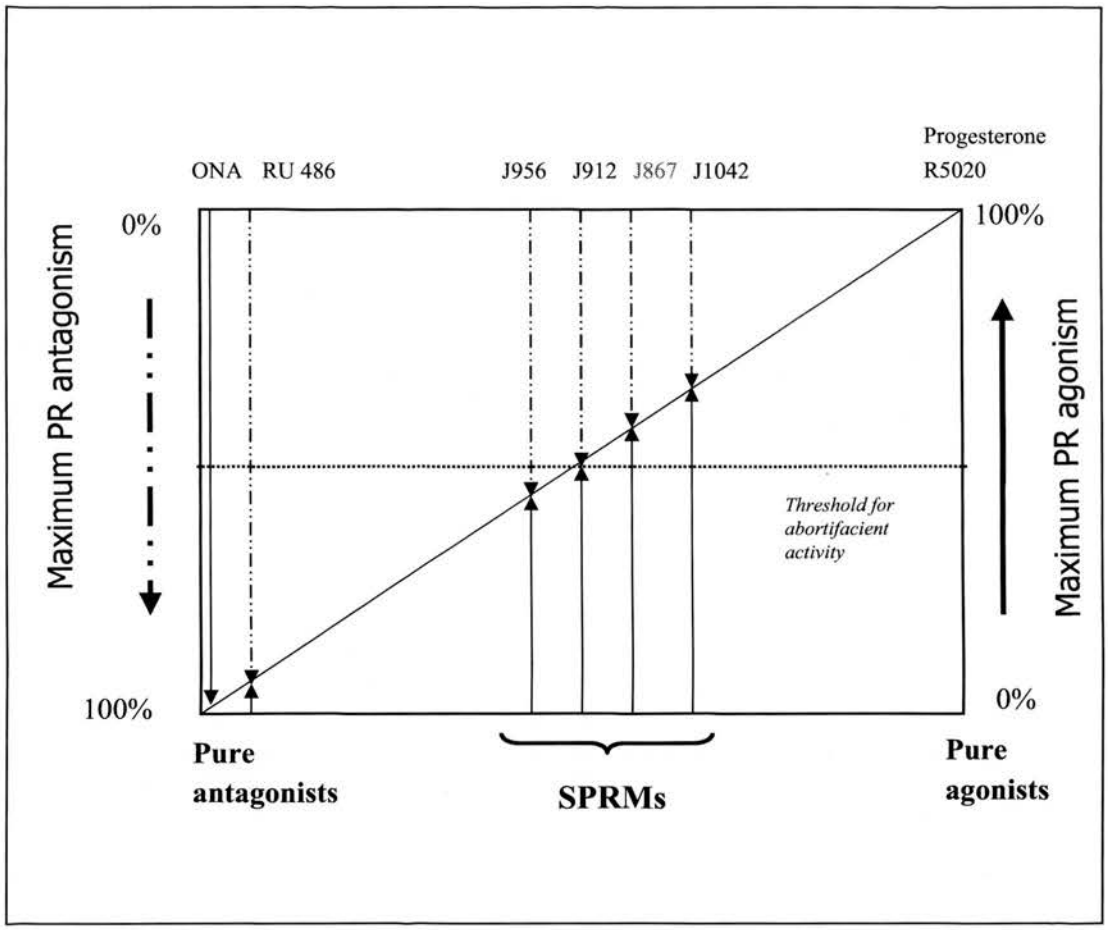


Figure 1.8. Spectrum of synthetic PR ligands ranging from progesterone agonists to antagonists

PR agonist and antagonistic properties of major 11β-benzoxime-substituted progesterone receptor modulators and progesterone antagonists; ONA, onapristone; RU486, mifepristone; J867, asoprisnil (Derived with permission from:
Schubert et al., Seminars of Reproductive Medicine 2005; 23: 58-73)

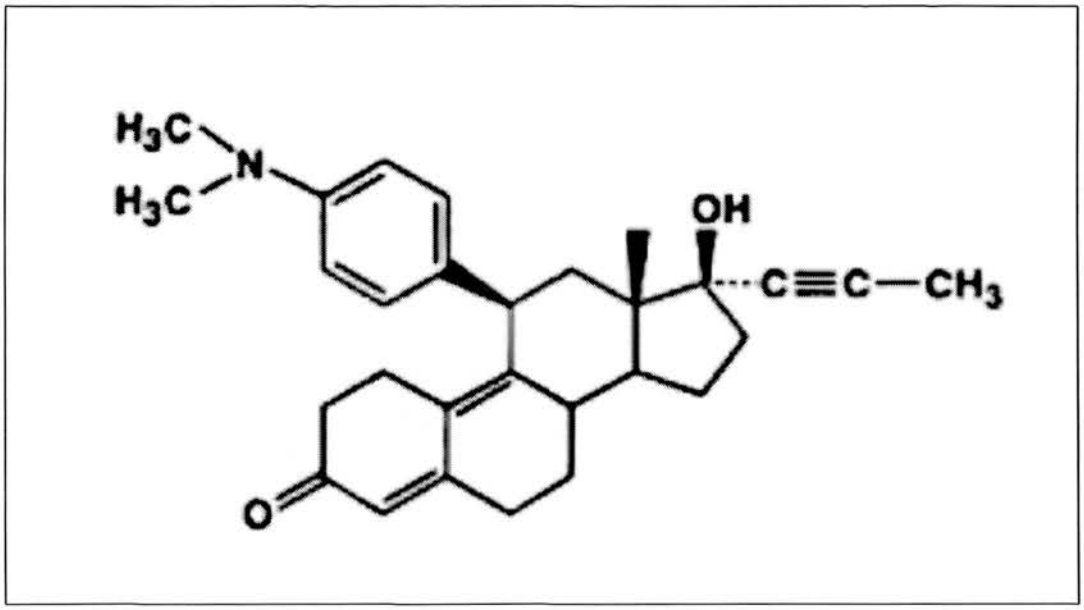


Figure 1.9. Chemical structure of mifepristone (RU486)

11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-propinyl-4,9-estradiene-3-one is a beta-aryl-substituted, 19-nortestosterone-derived compound

Recent randomised placebo-controlled trials with mifepristone in women with symptomatic fibroids have demonstrated a decrease in leiomyoma volumes and a significant reduction in menstrual blood loss with the effect of increased haemoglobin after treatment for three months[107,108]. Mifepristone has also been suggested as an adjunct to progestin-only contraception with the aim to reduce the occurrence of bleeding irregularities[109,110].

1.2.3.2. Other progesterone antagonists (PAs)

Mifepristone is currently licensed for clinical use in termination of pregnancy and ulipristal (CDB-2914) for emergency contraception[111]. A number of other PAs have been developed including onapristone, Org 31710, Org 33628, ZK 137 316, ZK 230 211 and others. Pre-clinical[112-115] and clinical studies[116] on the effects of these PAs have been published. In rhesus macaque monkeys, the effects of ZK 137 316 and ZK 230 211 have been studied with findings of dose-dependent suppression of menstrual and ovarian cyclicity[113,115]. Anovulation and amenorrhoea was demonstrated with

higher doses of ZK 137 316 and all doses of ZK 230 211, indicating a more potent P antagonistic activity of ZK 230 211. Studies in macaques have been conducted with ZK 230 211 administered via an intrauterine system (IUS) with similar results[114]. These agents have been suggested for consideration as contraceptives and for suppression of menstruation in humans. The use of ZK230 211 administered via an IUS in humans with a view to its potential in contraception and management of benign gynaecological conditions has been evaluated in one pilot study[116].

As unscheduled breakthrough bleeding is the most common reason for discontinuation of progestin-only contraception, PAs have been proposed to ameliorate this side effect[117]. Org 31710 administered monthly in addition to a desogestrel-only contraceptive pill appeared to regularize the bleeding pattern[118]. However, this effect has not been consistently demonstrated with other PAs. Moreover, the effect on the contraceptive efficacy also remains to be clarified[119].

1.3. Selective progesterone receptor modulators (SPRMs)

1.3.1. Discovery

The discovery of mifepristone (RU 486) was only the starting point of the development of a variety of PR ligands. Initially, the aim was to find a compound with more profound P antagonistic potency but less antiglucocorticoid activity. In these early stages, the envisaged applications were fertility control and treatment of breast cancer[120,121]. Very soon, the major therapeutic potential of PAs for the management of benign gynaecological conditions became apparent, when the endometrial antiproliferative effect resulting in endometrial atrophy and amenorrhoea was demonstrated initially in non-human primates and rabbits[122,123] and later in women[124]. The disadvantages of PAs at this point included the unopposed oestrogenic effects, the high abortifacient activity and the antiglucocorticoid effects at higher doses. Consequent concerns regarding the administration of PAs led to the conceptualisation of a drug discovery program to develop a compound with partial PR agonistic and antagonistic properties in the assumption that this would eliminate the perceived disadvantages of PAs[125].

A large number of compounds were synthesized and subsequently submitted to a screening program to determine their binding affinity to PR and GR, their labour-inducing potential in guinea pigs and their PR agonist and antagonist properties. The latter were assessed qualitatively with the luteolysis inhibition assay in guinea pigs and then quantitatively with the McPhail assay in immature rabbits. In the luteolysis

inhibition assay, partial PA effects of a compound were evaluated based on the potential to suppress endometrial prostaglandin ($PGF_{2\alpha}$) secretion in non-pregnant guinea pigs, as uterine $PGF_{2\alpha}$ is responsible for luteolysis in this species[126]. For the McPhail assay, immature E primed rabbits are treated with a compound, and the progestogenic effect on histological endometrial changes is subsequently evaluated and scored on a scale of 0 - 4[127].

The lead compounds selected were the 11β-benzaldoxime-substituted estratriene derivatives J867 (asoprisnil (benzaldehyde-4-[(11b,17b)-17-methoxy-17-(methoxymethyl)-3-oxoestra-4,9-dien-11-yl]-1-oxime); Figure 1.10.), J912, J956 (asoprisnil ecamate) and J1042, classified as SPRMs.

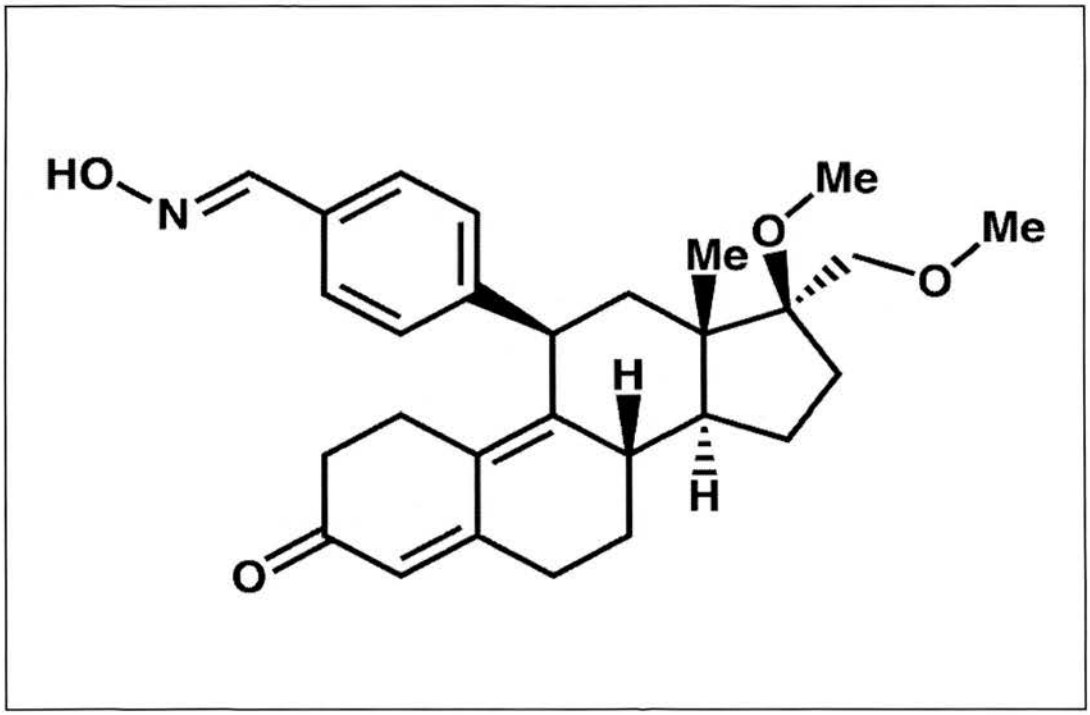


Figure 1.10. Chemical structure of asoprisnil (J867)

Benzaldehyde-4-[(11b,17b)-17-methoxy-17-(methoxymethyl)-3-oxoestra-4,9-dien-11-yl]-1-oxime is an 11β-benzaldoxime-substituted estratriene derivatives

They demonstrated high PR binding affinity and PR specificity with much reduced antiglucocorticoid activity. Their labour-inducing effects in guinea pigs were only marginal (asoprisnil, J956) or absent (J912, J1042). In the model tested, these J compounds and particularly asoprisnil and J1042 had the most PR agonistic potency. The hypothesis that the partial agonistic activity on the PR would result in a more favourable effect on the endometrium was confirmed in subsequent studies in non-human primates[128,129]. Due to its particularly pronounced PR agonistic properties and the absence of labour-inducing activity, asoprisnil was selected for further clinical development. Asoprisnil shows a high PR selectivity and a high degree of uterine selectivity[125].

1.3.2. Effects on endometrium

The discovery of the "endometrial antiproliferative effect" of PAs and SPRMs was an important milestone in their development[123,130]. It was first observed in rabbit and primate endometrium. The finding of endometrial atrophy induced by some PAs was unexpected for two reasons. None of the compounds were binding to ER and due to their PR antagonist activity, the endometrium would have been expected to exhibit unopposed oestrogenic effects. Instead, it demonstrated functional anti-oestrogenic effects.

As this phenomenon was further investigated, the importance of primate models became evident, as the endometrial morphology in rabbits exposed to PAs was different to primates. In rabbits, PAs induced similar inhibition of endometrial gland formation but stimulated endometrial stroma and myometrium[131]. In fact, the unique endometrial effects of PAs and SPRMs are specific to menstruating primates such as Old World monkeys and humans. Cynomolgus and rhesus macaques are particularly suitable models, as their endometrium is of striking similarity to the human with respect to hormonal regulation and morphological changes during the menstrual cycle[132,133].

Studies in non-human primates showed that PAs in both spayed and intact macaques induced endometrial atrophy with stromal compaction and inhibited mitotic activity. These effects were observed following administration of the PAs mifepristone (RU 486), ZK 230 211 and ZK 137 316. Studies with ZK 137 316 in the rhesus monkey also showed a dose-dependent degradation of the spiral arteries in the basal layer of the endometrium[131]. These profound morphological changes occurred irrespective of ongoing ovulatory cycles and in the presence of follicular phase oestrogen levels. The functional anti-oestrogenic effect was limited to the endometrium, whilst oviduct

and vagina remained unaffected. These studies provided the first evidence of the "endometrial antiproliferative effect" and suggested that PAs and SPRMs may be targeting the endometrium directly and possibly via the endometrial vasculature[123]. Some experiments also highlighted the importance of the dosages, as many effects were dose-dependent, and in the studies with the PA onapristone, the endometrial morphological findings at low and high doses were even divergent. Low doses of onapristone induced a similar pattern with suppressed endometrial maturation and gland formation, whilst treatment with high doses resulted in cystic endometrial hyperplasia[131].

During the drug discovery program to develop SPRMs with partial agonist activity, a study was conducted comparing the endometrial effects of J1042 with the PAs ZK 230 211 and ZK 137 316 in cynomolgus monkeys[134]. All three compounds induced the antiproliferative effect with decreased endometrial thickness, reduced mitotic activity in glands and stroma and increased stromal density. However, there were also interesting differences in the endometrial morphology following treatment with J1042 compared to the PAs, and for the first time, an SPRM was demonstrated to induce specific PR-mediated agonistic effects. The appearance of the glands was normal with no evidence of degeneration as seen after exposure to PAs. Additionally, the glandular epithelium showed signs of weak secretory activity such as glandular sacculation with subnuclear vacuolization and secretion. Differences were also seen in respect to the effects on the endometrial vasculature. Contrary to the PAs, J1042 did not cause hyalinizing degeneration of the spiral arteries but was more effective with regards to inhibition of endometrial angiogenesis. The intraluminal diameters of the spiral arteries were notably more reduced by J1042[125,131,132]. The "endometrial antiproliferative effect" with atrophy and increased stromal compactness was subsequently also demonstrated in studies with asoprisnil (J867) and J956 in cynomolgus monkeys[132]. As with PAs, these morphological features were observed in the presence of normal oestradiol levels and well-oestrogenized vaginal tissue indicating the high degree of endometrial selectivity. Similar to J1042, spiral artery degeneration did not occur with asoprisnil.

As crucial as these primate models have been for the classification and development of SPRMs, not all data are necessarily applicable to the human. Important differences between the responses of human and non-human primate endometrium to SPRMs have been observed and attributed to possible variances in the steroid receptor pharmacology. Most notably, formation of thick-walled endometrial spiral arteries following exposure to asoprisnil has been described in the human only[135]. Secretory

glandular changes in response to asoprisnil have also been demonstrated in the human but not in the cynomolgus monkey[132,135].

The endometrial changes following SPRM administration were consistent with mixed PR agonist and antagonist activity but had never been previously described. Some of the features had the potential to raise concern to the unfamiliar observer, and hence a panel of expert gynaecological pathologists convened and classified the specific effects of asoprisnil on endometrium. New diagnostic categories of "non-physiologic secretory effect" and "secretory pattern, mixed type" were developed and defined by weak glandular secretory effects, variable stromal effects mostly with increased compaction and clusters of thick-walled arterial vessels, and the number of mitotic figures[132,136].

1.3.3. Effects on menstrual bleeding

Consistent with the morphological finding of endometrial atrophy, PAs and also SPRMs including asoprisnil induce suppression of menstrual bleeding in non-human primates and in humans[125,131]. In a Phase I double-blind dose-escalation study, the effect of asoprisnil on 60 healthy premenopausal women was evaluated. They were administered different doses of asoprisnil from 5mg once daily to 50mg twice daily for 28 days commencing during the first four days of their regular menstrual cycle. As a result, cycle lengths were increased, and the onset of menstruation was significantly delayed with doses at or above 10mg once daily[137]. Suppression of menstruation with asoprisnil has been found to be reversible and not associated with the adverse systemic side effects of oestrogen deprivation[125,131]. The prolongation of the menstrual cycle in this Phase I study occurred even in the presence of a normal luteal phase and luteolysis indicating that the endometrium is directly targeted by asoprisnil[137]. Contrary to the experiences with continuous progestin treatment, which commonly results in unscheduled bleeding episodes particularly during the initial months of treatment[92], spotting or breakthrough bleeding was rarely reported with asoprisnil.

1.3.4. Effects on other clinical conditions

Asoprisnil has been the first SPRM to reach an advanced stage of clinical development for the treatment of benign gynaecological conditions such as symptomatic uterine fibroids and endometriosis[128,138,139].

Phase II studies in women with uterine fibroids reaffirmed the observation that asoprisnil induces reversible amenorrhoea. In a double-blind, placebo-controlled study, doses of 5, 10 or 25mg of asoprisnil were administered orally to women with uterine fibroids for 12 weeks. Duration and intensity of uterine bleeding were significantly reduced in a dose-dependent manner, and no episodes of unscheduled bleeding were reported. In addition to the suppression of both normal and heavy menstrual bleeding, a reduction in the volume of the largest fibroid was demonstrated resulting in a dose-dependent improvement of pressure symptoms such as bloating and pelvic pressure[138,139].

Phase II studies with asoprisnil have also been conducted in women with pelvic pain due to endometriosis. The rationale for the use of asoprisnil in this condition was the presumed positive effect of tissue-selective inhibition of endometrial proliferation and suppression of endometrial bleeding by targeting the endometrial vasculature directly on the symptoms of endometriosis[128,139]. The finding of tissue-specific suppression of endometrial prostaglandin production in preclinical studies also appeared promising regarding the potential of asoprisnil to ameliorate endometriosis-associated pain[122,140]. In a randomised, placebo-controlled study, doses of 5, 10 or 25mg asoprisnil were administered for 12 weeks to women with a laparoscopic diagnosis of endometriosis who suffered moderate or severe pain. Indeed, all three doses significantly reduced non-menstrual pelvic pain and dysmenorrhoea compared to placebo[139].

Therefore, the potential of asoprisnil to become a novel compound for medical management of heavy menstrual bleeding, symptomatic uterine fibroids and endometriosis has been highlighted and emphasized by a favourable safety and tolerability profile in all clinical studies to date.

1.4. Aims

The data presented within this thesis are derived from a phase II multi-centre randomised double-blind placebo-controlled study of asoprisnil administered for 12 weeks to women with symptomatic uterine fibroids scheduled for hysterectomy.
The aims of the study were to thoroughly investigate the effects of asoprisnil on:

1 a Clinical symptoms (i.e. menstrual blood loss)
b Uterine artery blood flow
c Ovarian function
d Clinical safety and tolerability

2 Endometrial histomorphology

3 Endometrial proliferation markers and PTEN expression

To elucidate the mechanism of action of asoprisnil, further laboratory investigations were conducted to study the effects on:

4 Endometrial sex steroid receptor expression

5 Endometrial gene expression and local immune cell function

Ultimately, through enhanced understanding of its mechanism and added evidence of efficacy and safety, the aim was to further specify the potential of asoprisnil for future clinical applications.

CHAPTER 2

GENERAL MATERIALS & METHODS

2.1. The Clinical Study

2.1.1. Study design

A phase II multi-centre, randomized, double-blind, placebo-controlled study was designed to investigate the effects of asoprisnil in women with symptomatic uterine fibroids. The study design has previously been described in a publication[141].
Selection of the asoprisnil doses was based on previous phase I and phase II studies. These had shown asoprisnil to be safe and well-tolerated when used for 12 weeks at doses of 5mg, 10mg and 25mg. Doses of 10mg and 25mg asoprisnil and the treatment duration of 12 weeks were chosen, as they had been effective in suppressing uterine bleeding and in reducing fibroid as well as uterine volumes over a period of 3 months[142]. The study drug was administered to patients for 12 weeks.

2.1.2. Recruitment of patients

Recruitment of premenopausal women volunteers was carried out in four centres (Edinburgh, Southampton, Glasgow, Liverpool). The women targeted for this study had to be in good general health. They had presented for a consultation with a specialist gynaecologist due to fibroid-related symptoms such as heavy menstrual bleeding (HMB) and / or pelvic pressure. They were approached for recruitment into this study after a decision for surgical intervention for their symptomatic uterine fibroids had been taken and they were scheduled for hysterectomy.
Each patient had at least one intramural non-pedunculated, submucosal or subserous fibroid with a diameter of ≥ 2cm or multiple small fibroids with a uterine volume of ≥ 200cm^3 confirmed by transvaginal or abdominal ultrasonography. Menstrual cycles had to be regular but the cycle length could vary between 17 and 42 days in different individuals. Other inclusion criteria included age over 18 years, negative pregnancy test, a washout period of 2-12 months for hormonal therapies (Table 2.1.), serum FSH <30mIU/ml at commencement of the study, a normal cervical smear test and agreement to use double barrier method of contraception (condom / diaphragm / sponge plus spermicide) throughout the study until hysterectomy unless surgically sterile by bilateral tubal ligation or vasectomy of the partner. There was an absolute requirement for patients to have had a normal endometrial biopsy report based on an adequate specimen within three months of entering the study. Additional pelvic pathology such as follicular-type or hemorrhagic ovarian cysts >3.5 cm in diameter, complex ovarian cysts or endometriosis led to exclusion from the study.

Therapy	Minimum Washout Interval (Prior to Dosing)
Medroxyprogesterone acetate (Depo-Provera)	12 months
GnRH analogue - 3 month depot [leuprorelin acetate (Lupron), goserelin acetate (Zoladex)]	9 months
GnRH analogue - 1 month depot	6 months
Danazol (Danocrine, Cyclomen)	6 months
Nafarelin acetate (Synarel)	6 months
Contraceptive injection (Lunelle Monthly)	4 months
Norethindrone acetate (Aygestin, Norlutate)	2 months
Norethindrone	2 months
Oral contraceptives	2 months
Medroxyprogesterone acetate (Provera)	2 months
Other progestins	2 months
Oestrogen preparations	2 months
Progesterone preparations	2 months
Progesterone- or Levonorgestrel-releasing IUS (Mirena®)	2 months

Table 2.1. Minimum washout period after hormonal therapy

Minimum period patients were required to have discontinued hormonal therapy prior to randomization into this study; after stopping their therapy, they also had to have had two normal periods before commencing the study medication

GnRH=gonadotrophin-releasing hormone; IUS=intrauterine system

All patients voluntarily signed a full Informed Consent Form (Appendix I). The study was performed according to the ethical principles of the Declaration of Helsinki (1989 revision) and the protocol approved by the Institutional Review Board (Multicentre Research Ethics Committee) (Appendix II).

2.1.3. Study procedures

As part of the study protocol, patients attended 5 - 7 study visits, as detailed in table 2.2. In the event of any concerns or adverse reactions, additional visits were arranged as required. At the first visit, screening data were collected in clinic mostly by gynaecologists but occasionally by study nurses. Screening procedures (performed within 45 days prior to study commencement) included pelvic and breast examination, complete physical examination, cervical smear test by liquid based cytology, ECG, transvaginal ultrasound (TVU), pipelle endometrial biopsy, contraception counselling and clinical laboratory investigations – haematology, biochemistry, endocrine panel, lipid profile, coagulation screen, urinalysis and serum and urine pregnancy tests. Patients were supplied with menstrual diaries and sanitary products as well as containers for the twice weekly collection of the first voided urine in the morning (Table 2.2.).

After successful enrolment based on inclusion and exclusion criteria, patients were re-assessed at the beginning of their next menses and treatment initiated no later than the fifth day of the menstrual cycle. Ideally, study procedures were carried out the day before and certainly within 7 days of commencing the study drug. Colour Doppler imaging of the uterine artery blood flow was performed during days 20-23 of the preceding menstrual cycle and therefore required an additional study visit for some patients. All patients were contacted by telephone after 4 weeks of treatment to enquire about any adverse events and to remind them about the requirements to use contraception and to continue their twice weekly urine collection. Patients attended for a visit after 8 weeks for review and for a thorough assessment after 12 weeks, just prior to or on the day of their hysterectomy (Table 2.2.). The hysterectomy was performed within 24 hours of the final dose of study drug. The study drug was supplied at the beginning of treatment with a further supply at week 8. On the occasions of the week 8 and week 12 visits, used and unused study medication blister cards were returned for verification of medication compliance. Six weeks after surgery, patients returned to the study site for a follow-up safety evaluation (Table 2.2.).

2.1.4. Randomization

A randomized double-blind design was applied to this study in order to minimize selection and subject bias. On the day prior to commencing treatment, patients received sequential subject numbers in ascending numerical order encoding the assignment to one of three treatment arms according to a randomization schedule generated prior to the study by the statistics department at TAP Pharmaceuticals Inc. The sealed box kits containing identical blister cards of study medication with 32 tablets were stored and dispensed by the pharmacies at the various study sites. Patients in three parallel dose groups in a 1:1:1 ratio self-administered daily doses of asoprisnil 10mg, 25mg or placebo orally. Patients and all study personnel including investigator, clinical research coordinator, monitors and pathologists were blinded to treatment groups throughout the study.

Table 2.2. Summary of study procedures

Participation in the study included between 5 and 7 study visits as detailed in this table:

- Screening visit – within 45 days of commencing the study drug
- Day-1 – Day prior to commencing the study drug within the first 5 days of the menstrual cycle
- Week 4 – Follow up by phone call on day 28
- Week 8
- Week12 – May be combined with hysterectomy visit
- OP - Day of the operation - Hysterectomy visit
- FU - Follow-up visit 6 weeks post-operatively

1 Clinical safety labs were to be obtained after fasting for at least 10 hours; they included blood samples for haematology, biochemistry, coagulation assays, lipid panel and endocrine panel; at week 8, they also included measurements of testosterone levels

2 Measurements of endometrial thickness, fibroid and uterine volumes

3 Colour Doppler imaging prior to commencing the study drug was done during days 20-23 of the preceding menstrual cycle; it therefore required a separate study visit for some patients

4 Collection of first voided urine of the day twice a week; samples were frozen by patients and then brought in at the next scheduled study visit

PROCEDURES	Screening	Day-1	Week 4	Week 8	Week 12	OP	FU
Informed consent	•						
Full history	•						
Contraception counselling	•	•	•	•	•		
Complete physical examination	•				•		•
Brief physical examination		•		•			
Pelvic and Breast examination	•	•			•		•
Cervical smear	•				•		
Vital signs	•	•		•	•		
Electrocardiogram (ECG)	•				•		
Clinical safety labs[1]	•	•		•	•		
Pregnancy test (serum and urine)	•	•		•	•		
Transvaginal ultrasound[2]	•	•			•		•
Colour Doppler imaging[3]		•			•		
Collection of urine samples[4]	•	•	•	•	•		
Endometrial biopsy	•						
Blood sample for P and E						•	
Hysterectomy						•	
Menstrual diaries and sanitary products dispensed	•	•		•			
Review of menstrual diaries		•		•	•		
Review of adverse events	•	•	•	•	•	•	•
Note concomitant medications	•	•	•	•	•	•	•
Assessment of symptoms		•		•	•		
UFS-QOL questionnaire		•			•		
Drugs dispensed		•		•			
Review of compliance				•	•		

2.2. Laboratory studies

2.2.1. Tissue collection

After removal of the uterus in the operating theatre, the unfixed specimen was placed on ice and taken without delay to the local pathology laboratory. The specimen was oriented and a probe inserted through the external os of the cervix to define the position of the cavity. The uterus was opened using a long-bladed knife along the plane of the probe. The opened specimen was then placed in an adequate volume of 4% neutral buffered formalin (NBF) and allowed to fix overnight. For large specimens, parallel parasagittal slices 2cm in thickness were made to permit adequate fixation. The following day, the specimen was sampled for routine diagnostic reporting and additional blocks taken for study assessment. Study blocks included full thickness endometrium with underlying myometrium. All study samples were routinely wax-embedded using the Tissue-tek embedding station and 5μm sections prepared. Endometrial assessment was carried out by microscopic examination of haematoxylin-eosin stained sections. Phase of cycle was assessed using the updated conventional descriptive Noyes criteria of the normal menstrual cycle as described in Blaustein's Pathology of the Female Genital Tract[143]. Additional samples from the endometrium were placed in RNA stabilization solution, stored overnight at 4°C and then transferred to a cryotube to be frozen at -70°C for subsequent RNA extraction.

2.2.2. Immunohistochemistry

All antibodies were tested individually at a range of dilutions and different antigen retrieval conditions to define the protocol resulting in the least background with the highest specific staining. With every immunohistochemical staining procedure, negative controls were included replacing the primary antibody with a non-immune immunoglobulin G (IgG) of a matched antibody concentration and from the same species as the primary antibody. In some cases, the primary antibody was replaced with antibody diluent only. Positive controls of samples with a known expression of the protein of interest were also included with every staining procedure. Whenever possible, sections for direct comparison were stained in the same immunohistochemical run.

Protein of interest	Primary antibody	Dilution	Negative Control
Phosphate and tensin homologue (PTEN)	Monoclonal Mouse anti-PTEN antibody (NCL-PTEN), Novocastra, Newcastle, UK	1:600	Mouse IgG1, Sigma, Poole, Dorset, UK
Oestrogen receptor-α (ERα)	Monoclonal Mouse anti-ERα antibody, Clone 1D5 DAKO, Cambridge, UK	1:1200	Mouse IgG, Vector Laboratories, Peterborough, UK
Oestrogen receptor-β (ERβ)	Monoclonal Mouse anti-ERβ1 antibody Serotec, Oxford, UK	1:40	Antibody diluent
Progesterone receptor (PR)	Monoclonal Mouse anti-PR antibody Novocastra, Newcastle, UK	1:400	Mouse IgG
Glucocorticoid receptor (GR)	Monoclonal Mouse anti-GR antibody Novocastra, Newcastle, UK	1:40	Mouse IgG
Androgen receptor (AR)	Monoclonal Mouse anti-AR antibody (F-39) Novocastra, Newcastle, UK	1:360	Mouse IgG
Cluster of differentiation CD56	Monoclonal Mouse anti-CD56 antibody, Zymed Laboratories, Cambridge, UK	1:200	Mouse IgG1

Table 2.3. Primary antibodies used for automated immunohistochemical staining in the Bond-X Immunohistochemistry Staining System

2.2.2.1. Antigen retrieval

Antigen retrieval was required to break cross links formed during the fixation process and to allow the antibodies access to previously masked epitopes on the proteins of interest. 5μm paraffin embedded tissue sections were de-waxed in Histoclear for 10 minutes before rehydration in descending grades of alcohol to distilled water (dH_2O). The slides were washed with 0.01M phosphate buffered saline (PBS) and pressure-cooked in 0.01M sodium citrate (pH 6) for 5 minutes at setting 2/high for antigen retrieval. The tissue sections were then cooled for 20 minutes.

2.2.2.2. Immunostaining

Most of the immunostaining in this study was carried out using the Bond-X Immunohistochemistry Staining System. Once cooled down following the antigen retrieval, the tissue sections were transferred in dH_2O for automated staining. The Bond-X Staining System was loaded with the Bond detection system reagents (Bond Peroxidase Detection System with Counterstain) and the primary antibodies. For negative controls, the primary antibody was replaced with non-immune mouse IgG (MIgG or MIgG1) at a matched concentration or by antibody diluent (Table 2.3.). The automated staining process was then commenced according to the DEFINE protocol (3 hour Polymer Protocol, ID 10003, created by VISION-5T27W1UO\Bond Service – Appendix V). For immunostaining of PTEN and CD56, the 3 hour polymer REFINE protocol was used (ID 10004, created by BondPowerUser – Appendix VI). Following completion of staining, the sections were dehydrated in ascending grades of alcohol to xylene and finally mounted with pertex.

The remaining immunohistochemical staining (Ki-67 / PH3) was performed on the bench according to the protocols described within section 5.2.1.

2.2.2.3. Scoring and analysis of immunoreactivity

To assess intensity and frequency of immunostaining in endometrium, a histoscore[144] was applied to each tissue compartment of surface epithelium, glandular epithelium, stroma, endothelium and perivascular cells. For each tissue compartment, the staining intensity was categorized into 0=no immunostaining, 1=mild immunostaining, 2=moderate immunostaining and 3=strong immunostaining. This score was then multiplied by the percentage of cells showing this particular intensity of immunostaining in graduations of 10 (10%, 20% etc. up to 100%) giving an overall score between 0 and 300. Initially, two independent observers blinded to the treatment groups carried out the scoring. If their score varied by more than 50, the

section was double-scored jointly by both observers with a double-headed microscope until a final score was agreed on. Otherwise an average was taken of the two initial scores.

Due to the very scanty staining pattern, a histoscore was not considered to be appropriate for assessment of proliferation markers Ki-67 and PH3. Different methods were applied for scoring as described within section 5.2.2.

2.2.3. RNA extraction and Quantitative reverse transcription polymerase chain reaction (Q-RT-PCR)

2.2.3.1. RNA extraction

RNA was extracted using Trizol reagent according to manufacturers instructions as detailed below.

Endometrial samples were immersed in 1ml Trizol RNA isolation reagent and then homogenized with a hand held homogeniser. Following further incubation in Trizol for five minutes at room temperature, the samples were transferred to phase lock gel tubes (PLG tubes). 200µl of chloroform was added and mixed in for 15 seconds before incubating for three minutes at room temperature. 15 minutes in the centrifuge at 12,000 x g and 4°C allowed separation of the RNA containing aqueous phase. PLG tubes contain a gel, which forms a solid barrier between the aqueous RNA containing layer and the remaining solution. It was therefore possible to pour the upper aqueous layer into a fresh eppendorf tube without DNA or protein contamination. 500µl isopropanol was then added to this aqueous layer for precipitation of the RNA. The samples were briefly vortexed, incubated at room temperature for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was carefully removed and the pellet washed in 1.5ml 75% ethanol. After a further spin at 7500 x g for 10 minutes at 4°C, the ethanol was removed and the remaining pellet allowed to dry for 5-10 minutes before suspending in an RNA storage solution. Some endometrial RNA was subsequently put through Qiagen RNeasy mini kit columns for further purification. RNA samples were stored at -70°C until further use.

2.2.3.2. DNAse treatment and Quality assessment of RNA

Following extraction, RNA was subjected to deoxyribonuclease (DNAse) treatment using 1U DNAse 1 (amp grade) / µg RNA in DNAse reaction buffer for 15 min at room temperature to remove genomic DNA contamination. DNA can otherwise lead to false positive results during taq-man real time RT-PCR. The reaction was stopped

by adding a final concentration of 2.5mM EDTA followed by heating to 99°C for 5 minutes, giving a final RNA concentration of 100ng/µl.
The quality of each individual RNA sample was checked with the Agilent RNA 6000 Nano Kit. Only samples of good quality were used for further analysis.

2.2.3.3. Reverse transcription

200ng of DNAse treated RNA was reverse transcribed in a buffered solution containing 5.5mM $MgCl_2$, 2.5µM random hexamers, 500µM of each deoxy-NTP, 0.4 U/µl ribonuclease inhibitor and 1.25 U/µl multiscribe reverse transcriptase. Samples were incubated for 60 min at 25°C, 45 min at 48°C and 5 min at 95°C. Negative controls were included in every run. An RT-negative control had template RNA but no multiscribe enzyme included, and an H_2O RT had nuclease-free water replacing the template RNA.

2.2.3.4. Taq-man real-time Q-RT-PCR

The method of taq-man real-time Q-RT-PCR detects and quantifies the emission of fluorescence from a reporter dye, which is produced during the amplification of target sequences from samples in real time. The technique uses forward and reverse primers to the sequence of interest along with a probe, which will specifically anneal between these two primers. Taq-man probes are labelled with two fluorescent dyes: at the 5' end with a reporter dye (all amplicons except 18S use FAM; 6-carboxyfluorescein) and at the 3' end with a quencher dye (TAMRA; 6-carboxytetramethylrhodamine). Prior to the start of the reaction, the quencher dye suppresses the fluorescence of the reporter dye as it is in close proximity. This process is known as FRET (Fluorescence Resonance Energy Transfer). When the target sequence is amplified during the PCR reaction, the polymerase enzyme AmpliTaq Gold DNA Polymerase first displaces the probe. Subsequently, due to the 5' fork-like structure dependent nuclease activity of AmpliTaq Gold DNA Polymerase, the probe is cleaved between the reporter and the quencher. Polymerisation of the strand continues as the probe fragments are displaced from the target. The quencher dye is separated from the reporter and can no longer suppress its fluorescence resulting in increased emission. It is the increased fluorescence, which is measured during each PCR cycle and which is directly proportional to the amount of target sequence amplified. The increasing fluorescence is plotted against the PCR cycle number (Figure 2.1.). Fluorescence is only detected if the target sequence of the probe is amplified during the reaction, and therefore detection of non-specific amplification is prevented.

Figure 2.1. Real-time Q-RT-PCR

Stage 1 Polymerization of primers and probes to the sequence of interest on template complementary DNA (cDNA); probe is intact and fluorescence is low as quencher dye (TAMRA) suppresses fluorescence of reporter dye (FAM)

Stage 2 Extension from the primer displaces the Taq-man probe from the cDNA

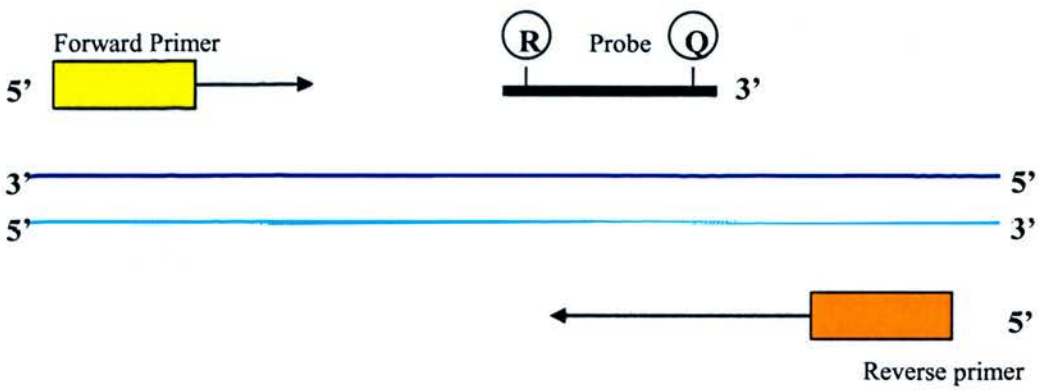
Stage 3 The displaced probe is cleaved by the nuclease activity of AmpliTaq Gold DNA Polymerase; reporter and quencher dye are separated, fluorescence increases and allows measurement of the PCR product generated

Stage 4 Polymerisation is complete

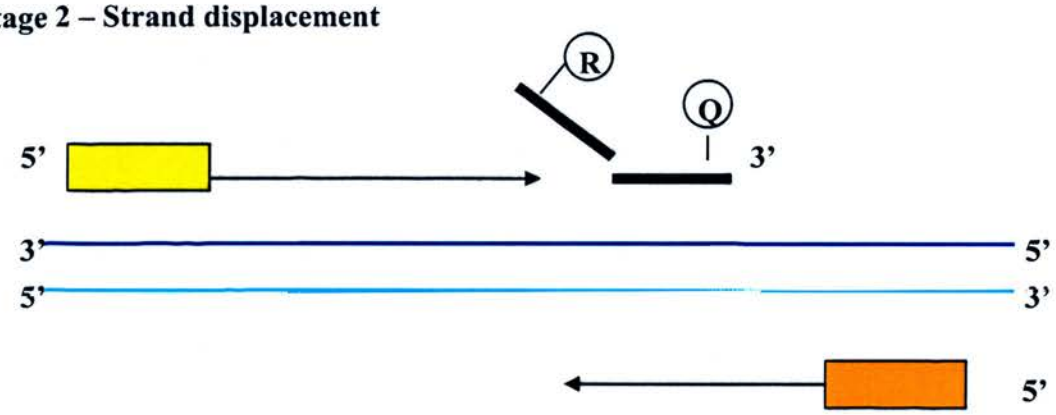
Adapted from Taq-man PCR reagent kit protocol

Stage 1 – Polymerization

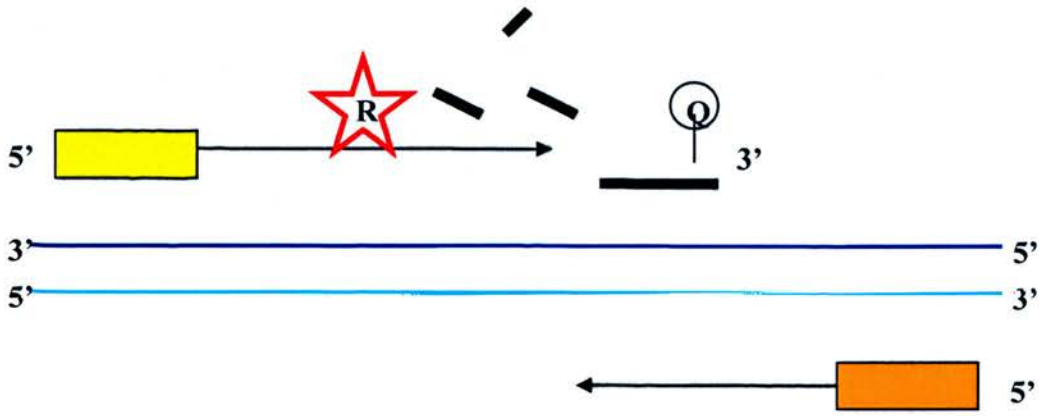
R = Reporter
Q = Quencher



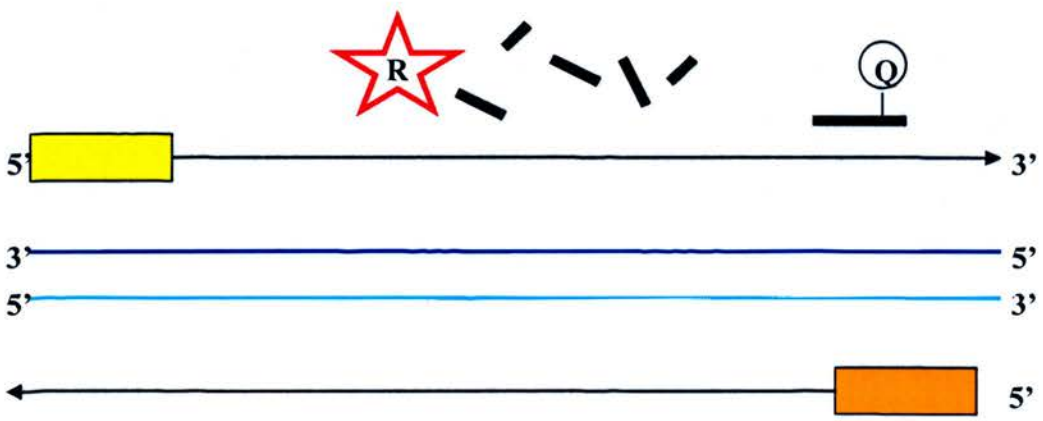
Stage 2 – Strand displacement



Stage 3 - Cleavage



Stage 4 – Polymerization complete



Primers and Probe		Sequence	Position on sequence	Accession number
ERα	Forward	TGATTGGTCTCGTCTGGCG	1523 -1541	NM_000125
	Reverse	CATGCCCTCTACACATTTTCCC	1602 -1624 (r)	
	Probe	TGCTCCTAACTTGCTCTTGGACAGGAACC	1572 -1600	
PR	Forward	CAGTGGGCGTTCCAAATGA	2151 -2170	NM_000926
	Reverse	TGGTGGAATCAACTGTATGTCTTGA	2209 -2233 (r)	
	Probe	AGCCAAGCCCTAAGCCAGAGATTCACTTT	2170 -2199	
AR	Forward	GTACCCTGGCGGCATGGT	951 -968	L29496
	Reverse	CCCATTTCGCTTTTGACACA	997 -1016	
	Probe	AGCAGAGTGCCCTATCCCAGTCCCA	970 -994	
GR	Forward	GCGATGGTCTCAGAAACCAAAC	4236 -4257	NM_000176
	Reverse	GCAGAGGATAACTTCCTCTGTAATCTC	4332 -4358	
	Probe	TCAGAGCCTCAGAACCTTCACTGCA	4300 -4325 (r)	

Table 2.4.A. Primer and probe sequences for amplification by Taq-man real-time Q-RT-PCR

Positions of the sequences within the cDNA are identified by the accession number; r denotes reverse strand

Primers and Probe		Sequence	Position on sequence	Accession number
ERβ1	Forward	CCTGGCTAACCTCCTGATGCT	1459 -1480	AB006590
	Reverse	CCACATTTTTGCACTTCATGTTG	1529 -1552 (r)	
	Probe	AGATGTTCCATGCCCTTGTTACTCGCA	1499 -1525 (r)	
CD56	Forward	CTCCACCCTCACCATCTAT	574 -596	NM_181351.3 NM_000615.5 NM_00107668 2.2. *
	Reverse	TCGCCTGTAACCACACACT	622 -642	
	Probe	CAACATCGACGACGGCCGG	598 -617	
IL-15	Forward	CAGATAGCCAGCCCATACAAG	721 -741	NM_172174.1 UPLProbe #46
	Reverse	GGCTATGGCAAGGGGTTT	786 -803	
	Probe	ATGGCTGC	775 -782	

Table 2.4.B. Primer and probe sequences for amplification by Taq-man real-time Q-RT-PCR

Positions of the sequences within the cDNA are identified by the accession number; r denotes reverse strand; * there are 3 transcripts of the CD56 gene, which would all be detected by the primers

It is possible to measure both ribosomal 18S and specific amplicon levels in the same PCR well in order to normalise the Taq-man reaction for differences in the initial cDNA loading. The expression of ribosomal 18S remains constant in proportion to the amount of cDNA. The different reporter dyes (FAM on amplicon and VIC (chemical name unavailable) on 18S probe) emit fluorescence at different wavelengths. The changes in the level of target sequence are therefore determined by comparing the levels of FAM to VIC fluorescence for each sample.

Taq-man real time Q-RT-PCR was used to measure levels of ERα, PR, AR, GR, ERβ, CD56 and IL-15 in endometrium. The reaction mix contained final concentrations of Taq-man universal PCR master mix (1X), ribosomal 18S forward and reverse primers and probe (50nM) as well as forward and reverse primers (300nM) and probe for the sequence of interest (200nM). The sequences of the previously validated primer/probe sets and their location within the specified cDNAs are listed in Tables 2.4.A&B. 1μl of cDNA was added per 25μl reaction volume, and each sample was tested in duplicate. A control with water replacing cDNA was included in every run as well as the controls from the RT step. Plates were sealed with adhesive covers and the PCR reaction run on the ABI Prism 7900 using standard settings.

2.3. Analysis of data

Statistical analyses were performed using the SAS® System 8.2. All statistical tests were two-sided. For each variable, the Hochberg multiple comparison procedure was used, and the familywise type I error rate was controlled at the level of 0.05. A subject's data were included in the analysis of immunohistochemistry or Q-RT-PCR provided there was an evaluable result.

2.3.1. Classification of Placebo Subjects

For analysis of clinical outcomes as detailed in chapter 3, all patients randomized into the placebo group were included. In the final analysis of immunohistochemistry and Q-RT-PCR data, patients treated with placebo were only considered for comparison if they had undergone hysterectomy in the secretory phase of their menstrual cycle. Samples from the proliferative phase were excluded so that the physiological variation of variables between different cycle phases would not influence the results. Patients in the secretory phase were chosen, as the endometrial effects of the PRM asoprisnil were expected to be most evident in comparison to

endometrium exposed to P. Patients with an average cycle length were most likely to be in the secretory phase at the time of hysterectomy after 12 weeks of treatment. Classification into proliferative or secretory phase was done according to morphological assessments of endometrial samples form the uterine fundus, mid-corpus and isthmus. Three categories (1=normal quiescent, 2=normal secretory or non-physiologic secretory, 3=normal proliferative or nonphysiologic proliferative) were applied. If all samples from a patient, which were satisfactory for diagnosis, were categorized as 2 or a mixture of 1 and 2, the patient was classified as secretory. Likewise, if all samples suitable for analysis were categorized as 3 or a mixture of 1 and 3, the patient was classified as proliferative. Samples from patients not meeting these criteria would be excluded from further analysis.

2.3.2. Immunohistochemistry

For statistical analysis of immunohistochemical protein expression, descriptive statistics (mean, standard deviation, median, minimum and maximum) were provided for each treatment group. Each group of asoprisnil treated patients (10 and 25mg) was then compared to the subgroup of placebo / secretory subjects. For comparison of each variable, the Wilcoxon's rank sum test was performed and significance determined at 0.05 level using Hochberg's multiple comparison procedure.

2.3.3. Taq-man real-time Q-RT-PCR

The data from the taq-man real-time Q-RT-PCR were analysed by calculating the threshold cycle (CT) for each sample. The threshold cycle (range 0-40) during the PCR amplification is reached, when the accumulation of PCR product shows exponential growth and the PCR signal crosses a defined threshold. A low CT value indicates a high initial amount of the sequence of interest, as the product accumulates earlier during the PCR reaction. ΔCT is calculated as the difference between CT values for the specific amplicon and 18S, in order to normalize for the initial cDNA load. The ΔCT value is then related to an endogenous control reference resulting in a ΔΔCT value for each sample. The ΔΔCT values were summarized and each asoprisnil group compared to the placebo / secretory subjects using Wilcoxon's rank sum test with statistical significance determined at 0.05 level using Hochberg's multiple comparison procedure. The amount of target normalized to the reference and relative to the placebo / secretory subjects was calculated as 2**(-[Mdn(asoprisnil)-Mdn(placebo/secretory)]).

CHAPTER 3

CLINICAL SYMPTOMS, UTERINE ARTERY BLOOD FLOW AND OVARIAN ACTIVITY

The data presented in this chapter have previously been peer-reviewed and published in the manuscript:

Wilkens J, Chwalisz K, Han C, Walker J, Cameron IT, Ingamells S, Lawrence AC, Lumsden MA, Hapangama D, Williams AR, Critchley HO
Effects of the selective progesterone receptor modulator asoprisnil on uterine artery blood flow, ovarian activity and clinical symptoms in patients with uterine leiomyomata scheduled for hysterectomy
Journal of Clinical Endocrinology & Metabolism 2008; 93(12): 4664-71

The author's personal involvement in this part of the study was the recruitment and clinical care of the patients in the centre with the most cases (Edinburgh). This involved the performance of the screening procedures and all clinical examinations throughout the study period, the coordination of all clinical visits, study investigations and sample collections and the maintenance of the case report forms.

3.1. Introduction

Heavy menstrual bleeding (HMB) is a common complaint, which frequently prompts women to seek medical advice. It may be a debilitating symptom and in some cases even indicates major abdominal surgery in the absence of any pathology. In the United States, HMB is the most common indication for 600,000 hysterectomies performed annually[145]. HMB is defined as blood loss of more than 80ml per month[146]. Not only is it rather impractical to accurately determine the amount of bleeding but more importantly, the subjective complaint correlates poorly with the actual blood loss[147]. For many reasons, the number of women seeking help for heavy bleeding seems to have risen. There is therefore a demand for improved and practical management options, particularly as many women with this complaint may be keen to preserve their fertility. The mechanisms of HMB are still not fully understood, and hence there remains an ongoing need to search for effective and acceptable therapies[3].

Uterine fibroids are an entity of benign gynaecological disease, which are known to affect menstrual bleeding pattern with a tendency to increase blood loss. Fibroids are benign smooth muscle tumours originating from the myometrium and clinically affect approximately 20-25% of all women of reproductive age[148,149]. The true incidence may be over 70%[150]. Whilst uterine fibroids may be entirely asymptomatic, HMB is one symptom commonly associated with the presence of fibroids.

Depending on their size and location within the uterus, fibroids may also cause pressure-related symptoms. For effective treatment strategies, it would therefore be desirable if there were an improvement of both bleeding pattern and symptoms due to fibroid size. Currently, medical management options are limited and their use restricted by side effects. As a result, uterine fibroids are with approximately 30% the second most frequent indication for hysterectomy in the United Kingdom[151,152].

The idea that fibroid-related symptoms may be managed by impairing uterine artery blood flow is the underlying principle of uterine artery embolization (UAE). With UAE, uterine arteries are occluded with Polyvinyl alcohol particles leading to ischaemic necrosis of fibroids whilst normal myometrial tissue is preserved via collateral blood vessels[153]. Fibroid tissue is rarely revascularized, and therefore the tumour mass shrinks with consequent resolution of symptoms. UAE has been shown to significantly reduce the amount of menstrual blood loss and also the severity of pelvic pressure and discomfort[154]. The outcome of UAE versus surgery in patients with symptomatic uterine fibroids has been evaluated to compare favourably[152]. Other less invasive methods of temporarily occluding uterine blood flow with the aim to manage the symptoms associated with fibroids have also been explored[155]. Whilst these techniques reduce uterine artery blood flow mechanically, it has been demonstrated that this can also be achieved pharmacologically. A decrease in uterine artery blood flow has been shown in women with a fibroid uterus with both the GnRH analogue leuprolide acetate and the progesterone receptor antagonist mifepristone and stipulated as a potential mechanism of action for the observed tumour shrinkage[156].

In the case of mifepristone, a reduction in fibroid volume may also be due to a direct antiprogestogenic effect. Evidence has been mounting to highlight a key role of progesterone (P) and the progesterone receptor (PR) in the development of uterine fibroids. Whilst traditionally, oestrogen has been regarded as the main hormone responsible for fibroid growth, more recent data from clinical and in vitro studies indicate that P also plays a pivotal role[84,85]. Up-regulation of both PR isoforms (PR-A and PR-B) has been shown in fibroid tissue compared to adjacent myometrium[157,158]. In addition, increased mitotic activity in fibroids was demonstrated during the luteal phase[159], and P increased the expression of the proliferation marker Ki-67 within fibroid tissue[160]. Synthetic progestins have been shown to reverse the effects of GnRH analogues on the size of uterine fibroids. This indicates that the clinical effects of GnRH analogues in women with a fibroid uterus may be due to the cessation of P secretion[161]. Fibroid volumes have also been demonstrated to decrease in small, uncontrolled, clinical studies with the

administration of mifepristone[162]. Collectively, these data suggest a stimulatory effect of P on the development of fibroids with the implication that PR antagonism may directly result in inhibition of fibroid growth.

The clinical effects of the selective progesterone receptor modulator (SPRM) asoprisnil (J867) with its partial and mixed PR agonist and antagonist properties have previously been evaluated in women with uterine fibroids. Doses of 5, 10 or 25mg were administered for three months to 129 patients with uterine fibroids in a multicentre, prospective randomized, double-blind, placebo-controlled, parallel-group study. The results demonstrated that asoprisnil suppressed uterine bleeding in 28%, 64%, 83% of subjects at 5, 10 and 25 mg, respectively, and reduced fibroid and uterine volumes[142]. The mechanism of action of these effects on symptoms and on fibroid size has not been elucidated.

There are many advantages of medical management of HMB and other fibroid-related symptoms over surgical intervention[3]. Even though endometrial resection and UAE carry a lower risk of morbidity than hysterectomy[152,163] and preserve the uterus, potential adverse effects on fertility and future pregnancies[164] still make them unsuitable options for women who have not completed their families. Successful pregnancies have been reported following UAE, but experience is still limited and the exact effect on placental function unknown[165]. Medical therapy is therefore the preferable option if further pregnancies are planned. It is also less expensive and less invasive. The main factors currently limiting its success are lack of evidence-based practice, poor compliance and unpleasant side effects[166].

The main classes of hormonal compounds used in drug therapy are progestogens and GnRH analogues. In both cases, compliance is limited by side effects. Even though systemic progestogenic side effects are minimal with the localized application of levonorgestrel via the intrauterine system (IUS), the issue of unscheduled breakthrough bleeding remains and is the main reason for discontinuation of treatment[93]. GnRH analogues exert their effects via suppression of ovarian hormones and are therefore associated with symptoms of hypo-oestrogenism. This limits their applicability and even during the six months they may be administered unopposed, hypo-oestrogenic side effects may become intolerable. Asoprisnil on the other hand, has been demonstrated to suppress menstrual bleeding profoundly with minimal breakthrough bleeding[137]. In the studies to date, the clinical and endometrial effects of asoprisnil have been observed in the presence of follicular phase oestrogen concentrations[142].

In this study, the clinical effects of asoprisnil in women with symptomatic uterine fibroids scheduled for hysterectomy were evaluated. The specific aims were:

1. To assess the effects on clinical symptoms associated with uterine fibroids such as menstrual blood loss, pelvic pain and discomfort and also fibroid-related quality of life
2. To perform ultrasonographic Doppler measurements of impedance (resistance and pulsatility indices) before and after treatment and assess the effects of asoprisnil on uterine artery blood flow as a possible mechanism of action
3. To determine ovarian activity during treatment by measuring the renally excreted metabolites of ovarian hormones
4. To depict the safety and tolerability profile of asoprisnil when administered for 12 weeks at doses of 10 and 25 mg

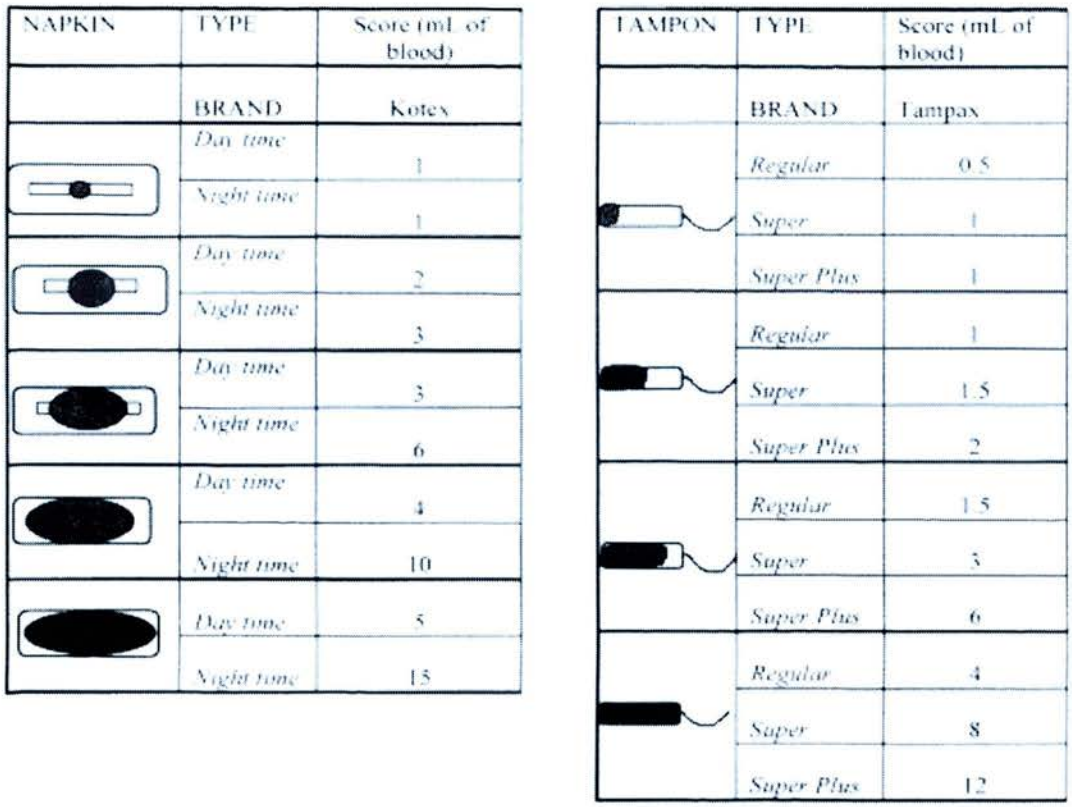
3.2. Materials & Methods

3.2.1. Study patients

Patients were recruited and enrolled as described in section 2.1. All patients were scheduled for hysterectomy due to symptomatic uterine fibroids and required to comply with all inclusion and exclusion criteria as cited in section 2.1.2. Once they had successfully undergone all screening procedures, they were randomised and administered the study drug for 12 weeks commencing within the first five days of a menstrual period.

3.2.2. Record of uterine bleeding

All patients were issued a daily diary and a menstrual pictogram (MP) at the screening visit, which they kept throughout the study period until hysterectomy. The diary was to record whether any uterine bleeding occurred including spotting. The use of any tranexamic acid or non-steroidal anti-inflammatory drugs (NSAID) was also noted. Whenever the amount of bleeding exceeded spotting and required more than a panty shield for protection, the MP was used to document and quantify the blood loss. Consistency of the record of uterine bleeding was enhanced by supplying patients with standardized sanitary products[167]. The MP reflected the type and number of sanitary products used (Tampax Regular, Super, and Super Plus, Kotex Maxi-Super and Maxi-Nighttime napkins) as well as the amount of blood soaked up by the products estimated by comparison of its appearance with the supplied pictogram (Figures 3.1 and 3.2.).



NAPKIN	TYPE	Score (mL of blood)
	BRAND	Kotex
	Day time	1
	Night time	1
	Day time	2
	Night time	3
	Day time	3
	Night time	6
	Day time	4
	Night time	10
	Day time	5
	Night time	15

TAMPON	TYPE	Score (mL of blood)
	BRAND	Tampax
	Regular	0.5
	Super	1
	Super Plus	1
	Regular	1
	Super	1.5
	Super Plus	2
	Regular	1.5
	Super	3
	Super Plus	6
	Regular	4
	Super	8
	Super Plus	12

Figure 3.1. Menstrual Pictogram (MP)

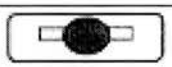
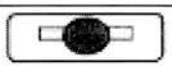
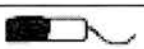
The MP supplies visual analogues of the appearance of standardized sanitary products (Tampax Regular, Super, and Super Plus, Kotex Maxi-Super and Maxi-Nighttime napkins) after use and the equivalent estimated blood loss;

patients recorded daily the number of sanitary products in each category enabling a calculation of estimated blood loss in millilitres

Figure 3.2. Example of daily diary with Menstrual Pictogram scores

Each day during the study, patients noted the absence or presence of bleeding requiring more protection than a panty shield; standardized sanitary products were used for protection and inspected after usage; they were compared to the visual analogues on the Menstrual Pictogram and the number of products in each category was noted each day; a total amount of blood loss was subsequently calculated for each day accordingly;

this example reflects a patient's diary from day –32 to day –4 during the screening period prior to commencing the study drug; it represents a menstrual blood loss of 42, 149, 86, 29 and 8 millilitres on days –32 to –28, respectively and 48 millilitres on day -4

Study day	Bleeding requiring more protection than panty shield	Menstrual Pictogram Match with Sanitary Product			Total Menstrual Pictogram score (millilitres of blood)
		Sanitary Product	Score (millilitres of blood)	Frequency	
-32	YES	 Night time	15	2	30
			4	3	12
					42
-31	YES	 Night time	15	3	45
			5	4	20
		 Super plus	12	7	84
					149
-30	YES	 Night time	10	1	10
			4	4	16
		 Super plus	12	5	60
					86
-29	YES		2	1	2
			3	1	3
		 Super plus	6	4	24
					29
-28	YES		2	1	2
		 Super plus	2	3	6
					8
-27 to –5	NO				
-4	YES		4	3	12
		 Super plus	12	3	36
					48

At each study visit, new diaries and sanitary products were dispensed and the previous diaries reviewed and collected by site personnel.
As described previously, the MP scores represent blood loss in milliliters[167]. For each patient, the MP scores were summed for the last full menstrual cycle before the randomization menses normalized to 28 days and for each 28-day treatment period. A total score was therefore calculated for baseline, weeks 1-4, weeks 5-8 and weeks 9-12. To evaluate the improvement in uterine bleeding, changes of MP scores from baseline to each month and from baseline to the final month was calculated and summarized. The number of days with bleeding requiring more protection than a panty shield were summarized according to the record in the diary for the pre-treatment cycle as well as for the three 28-day treatment periods as above. Changes from baseline to each month were calculated and summarized. The percentage of patients experiencing amenorrhoea throughout the study was calculated for each treatment group. Amenorrhoea was defined as having no days with bleeding or at least 42 days without bleeding requiring protection greater than a panty shield from the end of the pre-treatment menses through the end of dosing.

3.2.3. Assessment of fibroid-related symptoms

Fibroid-related symptoms except uterine bleeding over the month preceding treatment were assessed prior to dosing and after 8 and 12 weeks, respectively. They were recorded in the Leiomyoma Symptom Assessment Questionnaire (LSAQ) with four-point symptom scales (0=none or not applicable, 1=mild, 2=moderate, 3=severe) for bloating, pelvic pressure, dysmenorrhoea, urinary frequency and urinary incontinence. Nocturia was assessed as present or absent (Table 3.1.)[168]. For each of these symptoms, responses were summarized by 2-way contingency tables for each treatment group. For analysis, the number and percentage of patients in each treatment group with a decrease of at least 1 point was calculated for each symptom.
Prior to commencing the study drug and prior to hysterectomy, patients also completed the Uterine Fibroid Symptom and Health-Related Quality-of-Life questionnaire (UFS-QOL) with its subscales of concern, effect on activities, energy/mood, control, self-consciousness, sexual function and symptom severity[169].

Table 3.1. Leiomyoma Symptom Assessment Questionnaire

This Leiomyoma Symptom Assessment Questionnaire (LSAQ) was used to assess fibroid-related symptoms during the month preceding treatment and after 8 and 12 weeks, respectively

CATEGORY	Severity	Description
BLOATING - a feeling that your abdomen is swollen or enlarged	None	Symptom not present.
	Mild	Can be ignored.
	Moderate	Cannot be ignored but does not interfere with daily activities and/or medication or other treatment for bloating used occasionally.
	Severe	Cannot be ignored and affects concentration on daily activities and/or medication or other treatment for bloating used frequently.
PELVIC PRESSURE - the feeling that something is pushing down on your internal organs	None	Symptom not present.
	Mild	Can be ignored.
	Moderate	Cannot be ignored but does not interfere with daily activities and/or medication or other treatment for pelvic pressure used occasionally.
	Severe	Cannot be ignored and affects concentration on daily activities and/or medication or other treatment for pelvic pressure used frequently.
DYSMENORRHOEA - painful menses	NA	Not applicable; no menses since last visit.
	None	No pain during menses.
	Mild	Some loss of work efficiency.
	Moderate	In bed part of day, occasional loss of work.
	Severe	In bed 1 or more days.
URINARY INCONTINENCE - leakage of urine	None	Symptom not present.
	Mild	Can be ignored. Does not require protection.
	Moderate	Cannot be ignored but does not interfere with daily activities. Requires protection intermittently.
	Severe	Cannot be ignored and affects concentration on daily activities. Requires frequent protection for leakage.
URINARY FREQUENCY - feeling that you need to urinate frequently	None	Need to empty bladder <6 times during waking hours.
	Mild	Need to empty bladder 6-8 times during waking hours.
	Moderate	Need to empty bladder 9-10 times during waking hours.
	Severe	Need to empty bladder >10 times during waking hours.
NOCTURIA - feeling that you need to urinate during night hours	Absent	Need to empty bladder ≤1 time during the night.
	Present	Need to empty bladder ≥2 times during the night.

3.2.4. Sonographic assessment

Transvaginal ultrasound (TVU) (and abdominal ultrasound (TAS), if necessary) to assess the size of the uterus and of fibroids was performed at screening (within 45 days of study commencement) and prior to the start of dosing (during days 20–23 of the menstrual cycle prior to dosing). The volumes of the largest fibroid and the uterus were measured based on an estimation of the volume of an ellipsoid ($V=\pi LDW/6$ cm^3) where L, W, and D represent length, width and depth. A second TVU / TAS was performed after 12 weeks and the same measurements obtained to record any changes from baseline. The position of the fibroids within the uterus was not specifically recorded further to previous evidence that fibroid location does not necessarily correlate with the amount of uterine bleeding[170].

Colour Doppler imaging to determine blood flow of the main uterine arteries was carried out prior to the first study drug dose and after 12 weeks. Blood flow was estimated using two impedance indices: the resistance index (RI = systolic – end-diastolic peak velocity / systolic peak velocity) and the pulsatility index (PI = systolic – end-diastolic peak velocity / time-averaged maximum velocity)[171]. For each impedance index, two measurements were taken from both left and right arteries, respectively. The mean of the two measurements was calculated for each side, and for further analysis the mean of both sides was used[172]. In order to minimize variability, ultrasonographers were experienced in gynaecological procedures, and at each site all scans were performed by the same ultrasonographer throughout the study. All sites used the same colour Doppler imaging methods.

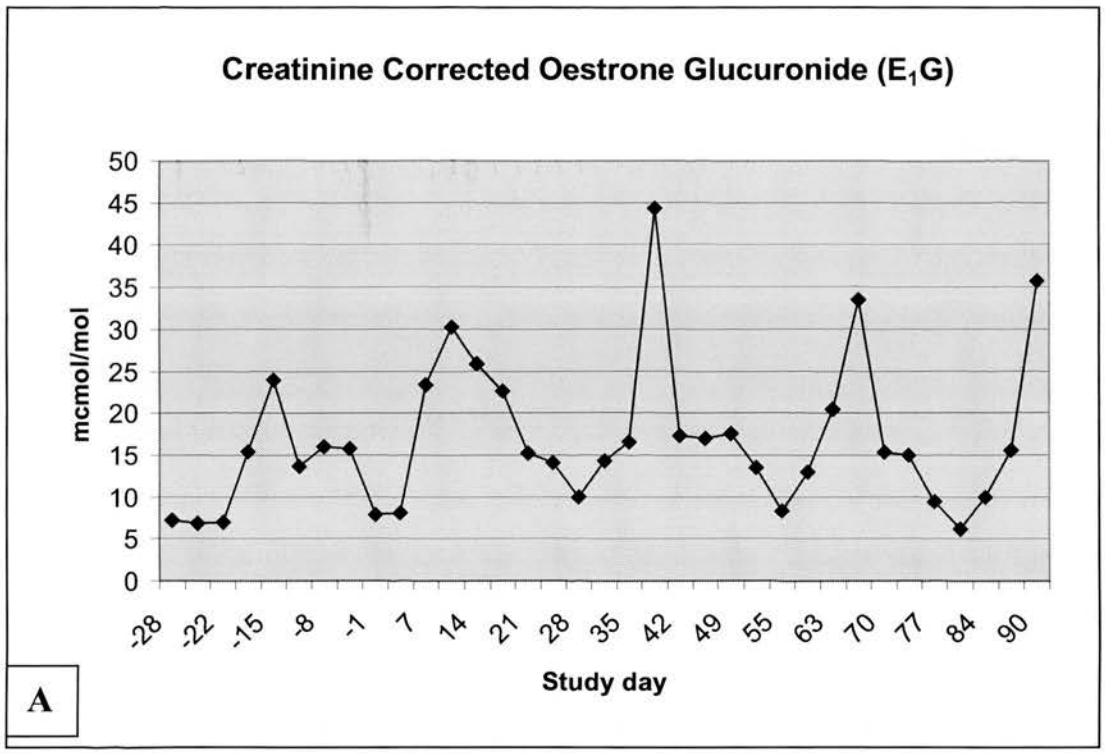
3.2.5. Ovarian activity

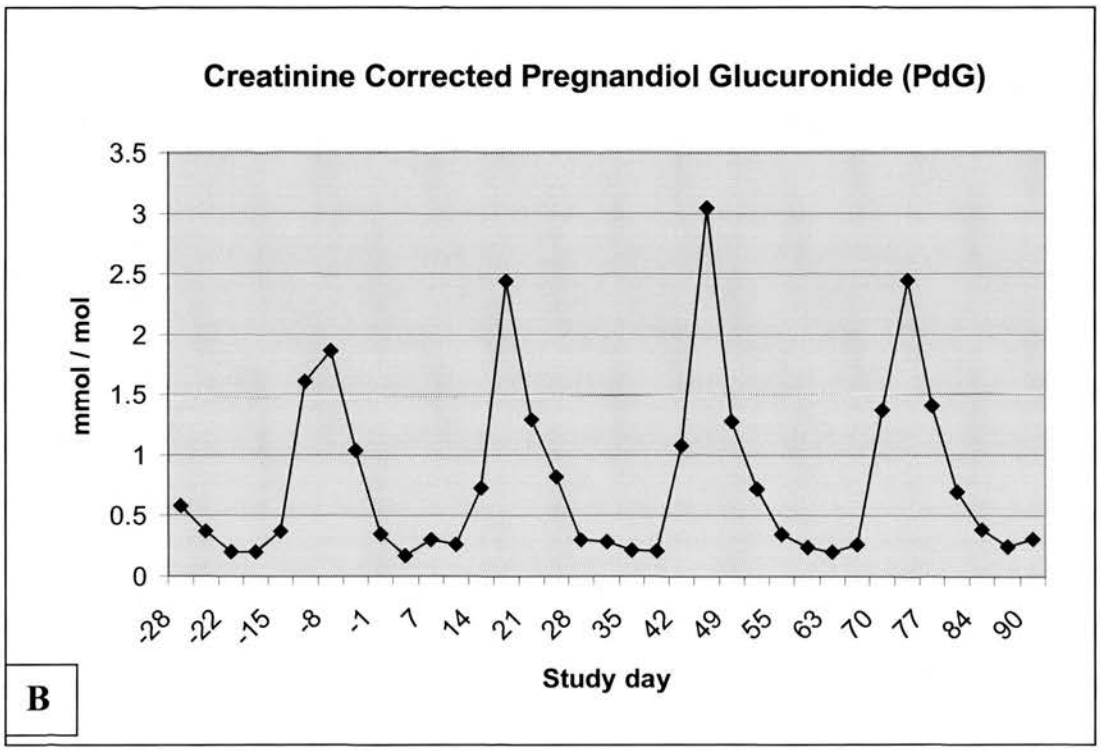
Patients were asked to collect a sample of the first voided urine of the day twice weekly throughout screening and treatment periods. These samples were kept in the freezer at -20°C for subsequent analysis. Urinary levels of the metabolites pregnandiol glucuronide (PdG) and oestrone glucuronide (E_1G) were measured using enzyme-linked immunoabsorbent assays (ELISA). Hormone concentrations were corrected for creatinine excretion and expressed as ratios of the creatinine concentration to urine volume[173] (Figure 3.3.). The results were used to determine ovarian follicular and luteal activity during the cycle prior to dosing and under the influence of study drug. Additionally, serum oestradiol and progesterone levels were also measured on the day of hysterectomy.

Figure 3.3. Examples of urinary ovarian hormone assays

A Example of a placebo treated patient's urinary creatinine oestrone glucuronide (E_1G) levels in mcmol/mol from study day –28 (screening period) to study day 90

B Example of a placebo treated patient's urinary creatinine corrected pregnandiol glucuronide (PdG) levels in mmol/mol from study day –28 (screening period) to 90


Creatinine Corrected Oestrone Glucuronide (E_1G)
mcmol/mol
50
45
40
35
30
25
20
15
10
5
0
-28
-22
-15
-8
-1
7
14
21
28
35
42
49
55
63
70
77
84
90
Study day
A


Creatinine Corrected Pregnandiol Glucuronide (PdG)
mmol / mol
3.5
3
2.5
2
1.5
1
0.5
0
-28
-22
-15
-8
-1
7
14
21
28
35
42
49
55
63
70
77
84
90
Study day
B

In order to evaluate the ovarian follicular activity during the 12-week treatment period, E_1G concentrations were compared to the pre-treatment follicular phase concentrations for each patient. The baseline ovarian function was the mean of the samples collected during the follicular phase of the pre-treatment cycle (i.e. the first 14 days of the screening menstrual cycle). To classify the follicular activity during treatment, the method previously described by Brown et al[174] was applied as follows:

1. Totally suppressed - E_1G <50% above baseline throughout treatment period
2. Partially suppressed - E_1G concentration ≥50% above baseline on at least one occasion but not meeting the definition of continued follicular activity
3. Continued - E_1G ≥50% above baseline on at least 2 occasions, separated by at least 13 calendar days during which there were no E_1G concentrations ≥50% above baseline

Number and percentage of patients belonging to each category as well as plots of E_1G concentrations during treatment over time for each patient, expressed as percentages of baseline level, were presented for each treatment group.

Urinary PdG levels were used to define evidence of luteal activity (ELA) versus no evidence of luteal activity (NELA). For each patient, each 4-week period during treatment (week1-4, week 5-8, week 9-12) was classified as having ELA or NELA with the aid of two different algorithms[175]:

Algorithm 1: A PdG concentration was considered ELA if it was at least 3 times as high as the minimum 3-concentration moving average of the PdG levels over the past four weeks. The first concentration to be classified as ELA or NELA was from the earliest sample obtained on or after Day 11, if it was at least the fourth available sample since Day 1 (inclusive). For each 28-day period during treatment, a subject was determined as having NELA, if no concentration was considered ELA during that period.

Algorithm 2: In addition to the requirements for the definition of ELA in algorithm 1, a PdG level at least 3 times as high as the 3-concentration moving average of the previous four weeks also had to be greater than 0.5 mmol/mol creatinine to be considered ELA.

For each 4-week period, each treatment group, and each algorithm, 95% exact confidence intervals on the percentage of patients with NELA were calculated.

3.2.6. Safety parameters

Throughout the duration of the study, each patient was closely monitored for the occurrence of adverse events (AEs). Physical examinations including check of vital

signs and breast and pelvic examinations were carried out at regular intervals. Further safety evaluation was by laboratory parameters (haematology, biochemistry, endocrine and lipid determinations, coagulation assays, urinalysis), pregnancy tests and electrocardiograms (ECG). Endometrial thickness and the adnexae were assessed during all TVUs as safety parameters. Patients who did not have both ovaries removed during the hysterectomy underwent a further TVU at the 6-week follow-up visit in order to inspect the remaining ovaries.

3.2.7. Data analysis and statistical methods

For the purpose of data analysis, each group of patients treated with asoprisnil (10 and 25mg) was compared to those who had received placebo and were in the secretory phase of their menstrual cycle at the time of hysterectomy. Pairwise comparisons within the framework of analysis of covariance (ANCOVA) models were used for assessments of change in RI, PI, MP scores, number of days with bleeding and UFS-QOL scores. The ANCOVA models for RI and PI included factors of treatment and investigator as fixed effects and baseline value as a covariate. In addition, a paired t-test was performed for RI and PI on the change from baseline to final visit for each treatment group. The models for MP scores, number of days with bleeding, and UFS-QOL scores included treatment as a factor and baseline value as a covariate. Wilcoxon's rank sum test was used to determine percent change in volume of the largest fibroid and the uterus. Percentage of patients with amenorrhoea and improvement in fibroid-related symptoms were compared by Fisher's exact test. For efficacy endpoints, Hochberg's multiple comparison method was applied to control for pairwise comparisons at a significance level of 0.05. No statistical inference was performed on safety variables.

At the outset of the study, it was planned to include 15 patients per treatment arm. This sample size would have provided greater than 95% power to detect a 0.08 difference in RI between an asoprisnil group and the placebo group using a two-tailed two-sample t-test with a common standard deviation of 0.05 (with a 0.05 significance level). The study was closed with a total of 33 patients due to recruitment delays. With 11 patients per group and the same assumptions as above, the power to detect a 0.08 difference in RI was 94%.

3.3. Results

3.3.1. Demographic information

A total of 33 patients were enrolled in this study at the four investigative sites (Edinburgh – 19, Southampton – 11, Glasgow – 2, Liverpool – 1). Thirteen patients underwent the screening procedures but then failed to meet the inclusion criteria. Ten, 12 and 11 patients received placebo, asoprisnil 10mg and 25mg, respectively, following assignment to each treatment group according to a 1:1:1 randomization scheme. The study was completed by all 33 patients who underwent 12 weeks of treatment, the scheduled hysterectomy and a follow-up assessment six weeks postoperatively (Figure 3.4.).

The majority of patients were Caucasian (87.9%) and the mean age across all groups was 43.9 years (range 35 to 52 years, inclusive). Treatment and placebo groups were well matched in terms of race, age, height, and weight (Table 3.2.). Drug compliance was calculated by comparing the number of study treatment days with the number of tablets taken according to the returned medication blister cards. Patients in all groups were at least 93% compliant, some patients had taken more tablets than expected, and their compliance therefore exceeded 100% (Table 3.3.). No patients developed withdrawal criteria during the study. No patient received the wrong treatment or an incorrect dose.

3.3.2. Uterine bleeding

Uterine bleeding was substantially decreased by treatment with asoprisnil. According to the MP scores, there was a large mean reduction in blood loss in the final month compared to baseline in both groups treated with asoprisnil (-154 ml after treatment with 10mg asoprisnil (95% confidence interval (CI) –276.2 to –57.6) and -215 ml with 25mg asoprisnil (95% CI –349.1 to –106.9)). These results indicate with 95% certainty that reduction of blood loss with administration of 10mg asoprisnil is at least -58 ml and at least –107 ml with 25mg asoprisnil. This was statistically significantly different from the mean increase of 13 ml in the placebo group (Figure 3.5. / Table 3.4.). These decreases were already apparent during the first four weeks of treatment.

The number of days with bleeding and percentage of patients with complete suppression of bleeding were calculated from the patient's diaries.

Figure 3.4. CONSORT diagram

Numbers of patients at different stages of the clinical study

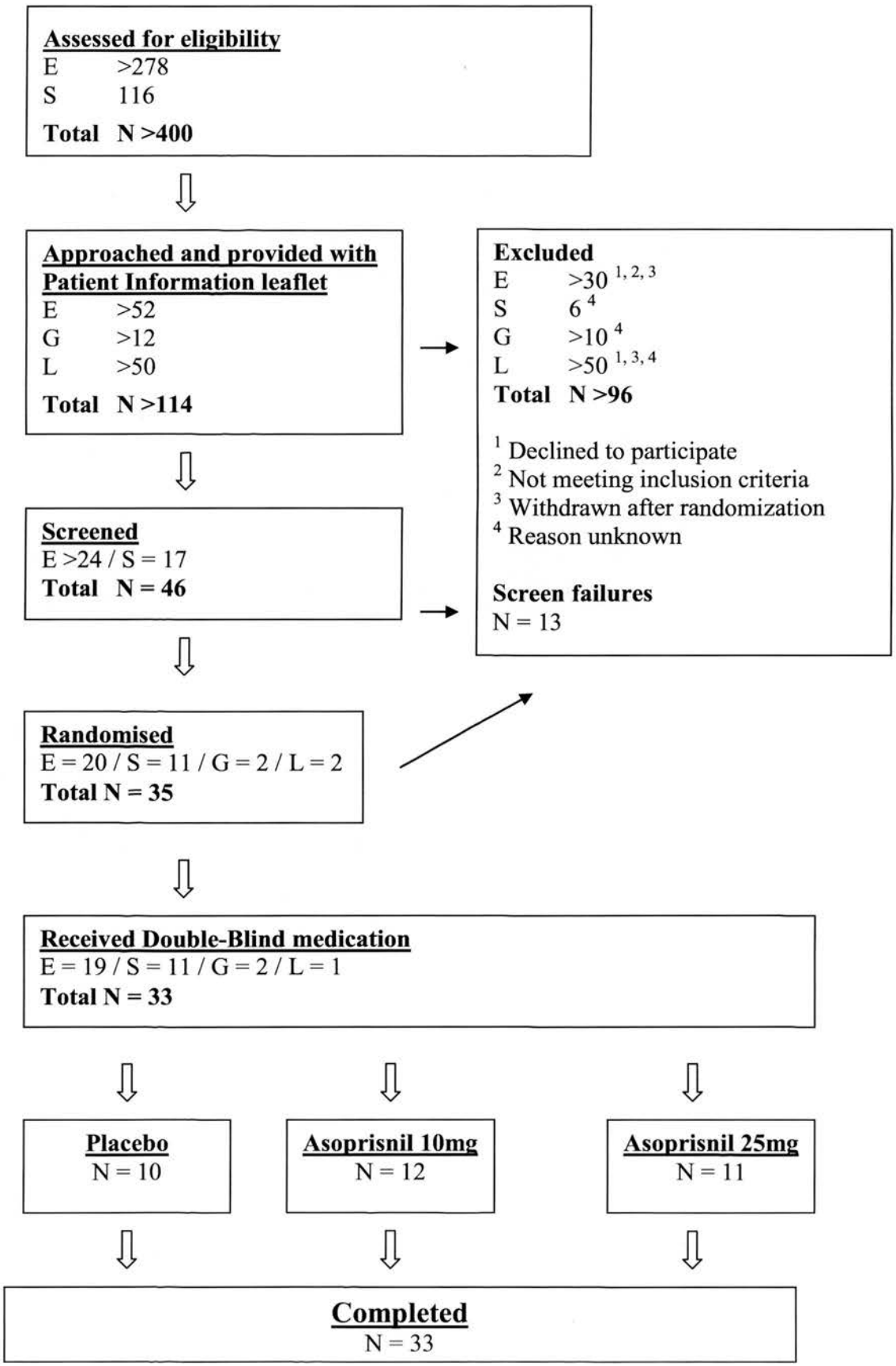
The figures for the numbers of patients whose notes were looked at to assess for eligibility and who were approached and given a patient information leaflet, are estimates based on best possible information at the time of assembling this diagram; numbers are divided into the information from the different study sites where appropriate

E = Edinburgh

S = Southampton

G = Glasgow

L = Liverpool


Assessed for eligibility
E >278
S 116
Total N >400
Approached and provided with Patient Information leaflet
E >52
G >12
L >50
Total N >114
Excluded
E >30 1, 2, 3
S 6 4
G >10 4
L >50 1, 3, 4
Total N >96
1 Declined to participate
2 Not meeting inclusion criteria
3 Withdrawn after randomization
4 Reason unknown
Screened
E >24 / S = 17
Total N = 46
Screen failures
N = 13
Randomised
E = 20 / S = 11 / G = 2 / L = 2
Total N = 35
Received Double-Blind medication
E = 19 / S = 11 / G = 2 / L = 1
Total N = 33
Placebo
N = 10
Asoprisnil 10mg
N = 12
Asoprisnil 25mg
N = 11
Completed
N = 33

Variable	Treatment Group			
	Placebo (N=10)	Asoprisnil 10 mg (N=12)	Asoprisnil 25 mg (N=11)	All Subjects (N=33)
Race (n [%])				
Black	1 [10]	2 [16.7]	1 [9.1]	4 [12.1]
Caucasian	9 [90]	10 [83.3]	10 [90.9]	29 [87.9]
Age (y)				
Mean (SD)	41.8 (3.6)	45.1 (3.5)	44.6 (6.0)	43.9 (4.6)
Range	37-48	39-50	35-52	35-52
Weight (kg)				
Mean (SD)	73.4 (11.7)	73.8 (17.7)	75.9 (11.8)	74.4 (13.8)
Range	54-89	45-105	60-96	45-105
Height (cm)				
Mean (SD)	165.3 (6.4)	164.3 (4.7)	165.6 (7.3)	165.1 (6.0)
Range	158-177	156-172	157-178	156-178

Table 3.2. **Demographic data at baseline**

Race, age, weight and height distribution across the three treatment groups at the time of screening

	Treatment Group		
% Compliance	**Placebo (N=10)**	**Asoprisnil 10 mg (N=12)**	**Asoprisnil 25 mg (N=11)**
Mean +/- SD	98.9 +/- 2.8	102.3 +/- 10.2	98.3 +/- 2.6
Minimum-Maximum	93-102	98-135	94-100

Table 3.3. Treatment Compliance

Compliance was calculated by dividing the number of tablets taken (according to the used and returned medication blister cards) by the number of study days (equal to the number of tablets expected to have been taken); some patients took more tablets than expected and had therefore compliance levels greater than 100%

	Treatment Group		
	Placebo (N=10) Mean ± SD	**Asoprisnil 10 mg (N=12) Mean ± SD**	**Asoprisnil 25 mg (N=11) Mean ± SD**
Baseline	213.0 ± 128.0	156.7 ± 103.8	217.9 ± 115.4
Final Month	225.6 ± 232.7	2.4 ± 4.9	2.5 ± 8.1
Change – Baseline to Final Month	12.6 ± 150.6	-154.3 ± 105.2	-215.4 ± 114.1
95% Confidence intervals (CI) for mean change	N/A	-276.2 to -57.6	-349.1 to -106.9
P-value[a] Comparison vs placebo	N/A	0.001[$]	<0.001[$]

Table 3.4. Menstrual pictogram (MP) scores

Mean changes of MP scores (in millilitres) from baseline to final visit in the three treatment groups (placebo, asoprisnil 10mg, asoprisnil 25mg)

[a] From ANCOVA model for change from baseline to final month with fixed effect of treatment and baseline score as a covariate

[$] Denotes statistical significance at 0.05 level using Hochberg's multiple comparison procedure

N/A = not applicable

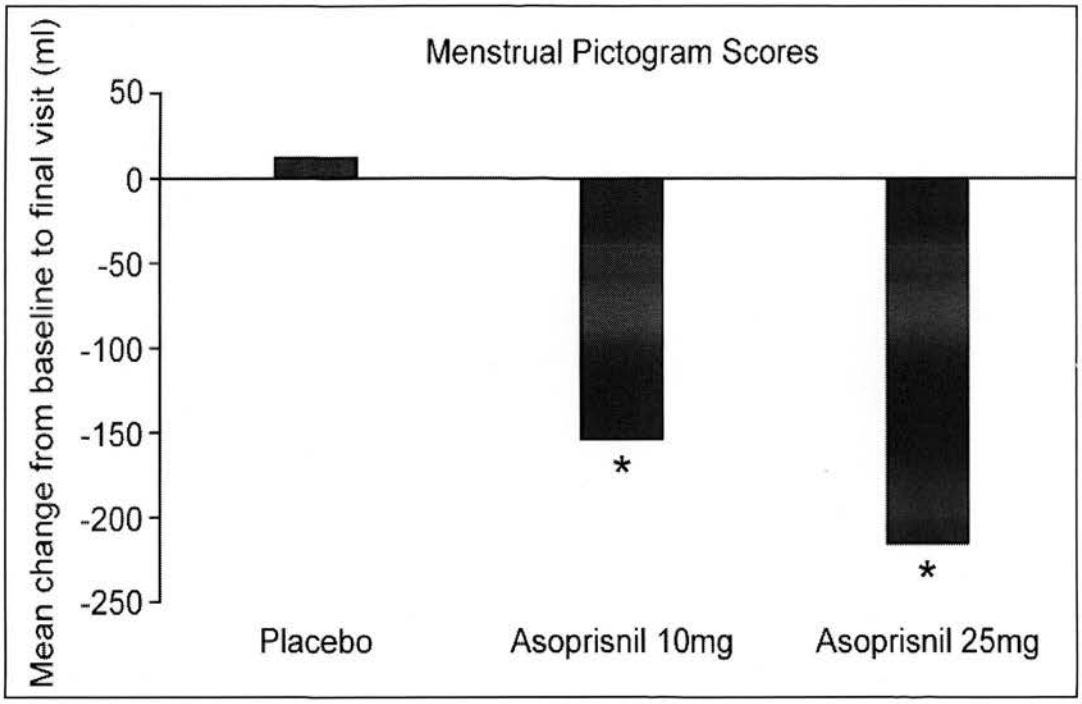


Figure 3.5. Menstrual Pictogram (MP) scores

Mean changes of MP scores (in millilitres) from baseline to final visit in the three treatment groups (placebo, asoprisnil 10mg, asoprisnil 25mg);
the significance of the difference of change from baseline between placebo and asoprisnil groups was determined using Hochberg's multiple comparison procedure at 0.05 level (*)

Analysis of the change from baseline during the treatment period showed that there was a statistically significantly larger decrease ($p<0.001$) in the number of days with bleeding for the asoprisnil groups compared to placebo.

Patients treated with asoprisnil 10mg and 25mg on average had bleeding of 7.0 and 8.0 days prior to dosing, which decreased to 1.2 and 0.2 days during the final month, respectively. The placebo group had a mean number of 7.3 bleeding days at baseline, which was unchanged in the final month. This difference between the groups treated with asoprisnil and the placebo group was statistically significant ($p<0.001$). The 95% confidence intervals for the change in the number of days with bleeding were –8.65 to –2.85 for asoprisnil 10mg and –10.28 to –5.2 for asoprisnil 25mg, indicating that the higher dose of 25mg asoprisnil leads to a reduction of at least –5 days.

The decrease in days with bleeding in the asoprisnil groups was evident during the first month and continued throughout the treatment period. Suppression of uterine bleeding, defined as having no days of bleeding requiring protection greater than a panty shield, occurred in 33% of patients treated with asoprisnil 10mg and 91% of those treated with 25mg. None of the patients in the placebo group showed suppression of uterine bleeding. The difference between the asoprisnil 25mg and placebo groups was statistically significant ($p<0.001$).

3.3.3. Fibroid-related symptoms

Evaluation of the LSAQs showed a statistically significantly greater proportion of patients with improvement in dysmenorrhoea at the final visit after treatment with asoprisnil than with placebo. Improvement in a symptom was defined as a decrease of at least one point on the symptom assessment scale. 100% improvement in the group treated with asoprisnil 25mg was already observed after eight weeks of treatment. At the final visit, bloating, pelvic pressure, urinary incontinence, urinary frequency and nocturia had improved but differences were not statistically significant in the asoprisnil groups compared to placebo (Figure 3.6.).

There was an improvement in UFS-QOL Total Scores from baseline to final visit for both asoprisnil groups and no change in the placebo group. The difference in change between asoprisnil groups and placebo-treated patients was statistically significant ($p=0.022$ for asoprisnil 10mg vs placebo; $p<0.001$ for asoprisnil 25mg vs placebo). These results indicate a significant effect of asoprisnil on quality of life in women with symptomatic uterine fibroids. In particular, there was statistically significantly greater improvement with asoprisnil for the scales of concern, activities, control and self-consciousness.

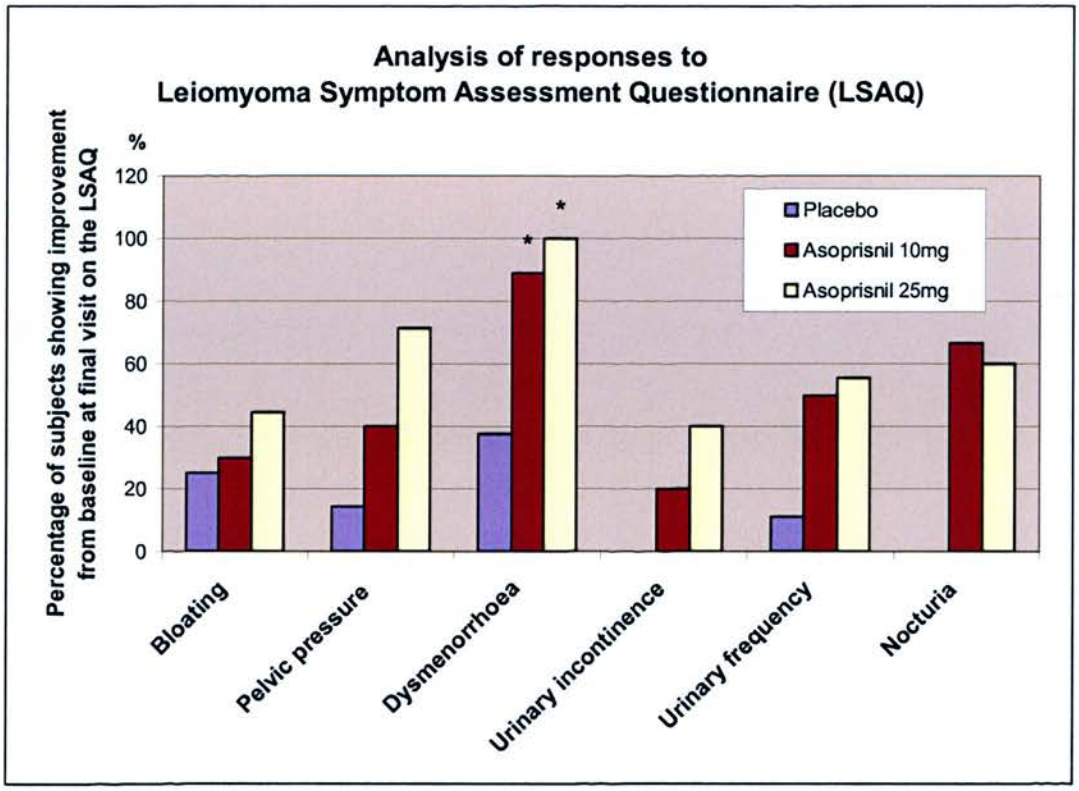


Figure 3.6. Analysis of responses to Leiomyoma Symptom Assessment Questionnaire (LSAQ)

Percentage of patients reporting improvement from baseline at the final visit on the LSAQ; symptom improvement is defined as decrease of at least one point on the symptom assessment scale; statistical analysis is by Fisher's exact tests; statistical significance is determined at 0.05 level using Hochberg's multiple comparison procedure (*)

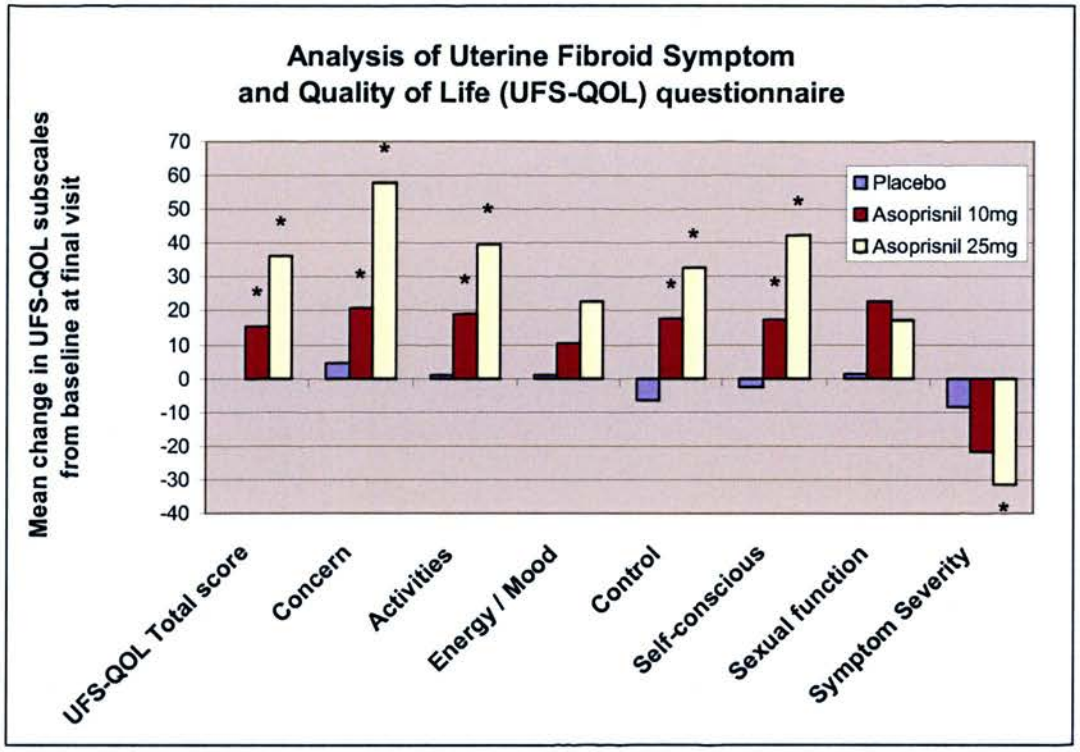


Figure 3.7. Analysis of Uterine Fibroid Symptom and Quality of Life (UFS-QOL) questionnaires

Mean changes in Uterine Fibroid Symptom and Quality of Life (UFS-QOL) total score and subscales (concern, activities, energy/mood, control, self-consciousness, sexual function, symptom severity) from baseline to final visit in the three treatment groups (placebo, asoprisnil 10mg, asoprisnil 25mg); the significance of the difference of change from baseline between placebo and asoprisnil groups was determined using Hochberg's multiple comparison procedure at 0.05 level (*); for symptom severity, a lower score indicates lower severity, for all other scales, a higher score corresponds to better quality of life

There was a trend towards greater improvement in energy/mood and sexual function even though the difference between changes in asoprisnil and placebo groups was not statistically significant. Reduced symptom severity was observed in both asoprisnil groups but only statistically significant after treatment with asoprisnil 25mg compared to placebo (Figure 3.7.).

3.3.4. Volume of largest fibroid and uterus

Treatment with 25mg asoprisnil resulted in a reduction of the volume of the largest fibroid with a median percent change of –25.8% from baseline to final visit. The decrease with 10mg asoprisnil was very minor (-0.4%), and there was an increase of 4.9% in the placebo group. The differences between asoprisnil groups and placebo were not statistically significant. There were also no statistically significant differences in the median percent change in volume of the uterus between asoprisnil and placebo groups.

3.3.5. Uterine artery blood flow

The change in RI between baseline and final visit was not statistically significantly different after treatment with either dose of asoprisnil compared to placebo. However, the increase in RI was statistically significant within the group administered 25mg asoprisnil (Figure 3.8. / Table 3.5.). The rise in PI from baseline to final visit in both asoprisnil groups was not statistically significant unlike the decrease following placebo treatment. The difference in change from baseline was statistically significantly different in both asoprisnil groups compared to placebo (Figure 3.9. / Table 3.5.). Overall, these results are indicative of moderately reduced uterine blood flow following administration of asoprisnil for 12 weeks.

3.3.6. Ovarian activity

Using two different algorithms as described above, luteal activity for three different time periods during treatment was calculated using urinary PdG levels. The mean PdG levels are presented in Table 3.6. Both methods of analysis gave similar results. These were summarized by indicating the number and percentage of patients showing no evidence of luteal activity (NELA) (Table 3.7.). During weeks 9-12, there was NELA in 70-80% of patients who had received 25mg asoprisnil compared with up to 20% in the placebo group. Even during weeks 1-4, there was evidence of

dose-dependent suppression of luteal activity with 10-20% of patients showing NELA in the placebo group compared to 33% following treatment with 10mg and 80-90% with 25mg asoprisnil.

Urinary E_1G levels (Table 3.6.) were used to assess follicular activity. Partial or total suppression was seen 22% of patients on placebo compared to 33% on asoprisnil 10mg and 60% on asoprisnil 25mg. On the other hand, continued follicular activity was seen in 78% of patients in the placebo group versus 67% and 40% in the asoprisnil 10mg and 25mg groups, respectively. These results indicate that asoprisnil has a dose-dependent suppressive effect on follicular activity.

Serum oestradiol (E) and progesterone (P) concentrations were measured after 12 weeks of treatment prior to hysterectomy. According to histological endometrial assessments, 80% of the placebo-treated patients underwent their hysterectomy during the secretory cycle phase. The mean E value was lower in the group on asoprisnil 25mg (0.5 pg/ml) compared to placebo and asoprisnil 10mg (1.34 pg/ml and 1.32 pg/ml, respectively). The mean P level was substantially lower in the groups on asoprisnil 10mg and 25mg (35.5 pg/ml and 5.7 pg/ml, respectively) compared to placebo (76.0 pg/ml).

3.3.7. Safety parameters

No serious adverse effect (AE) occurred in any asoprisnil-treated patient. No AEs led to discontinuation of the study. Even though over 90% of patients in each group experienced at least one AE during the treatment period, the majority of these events were mild or moderate in severity. The timing of many AEs indicated them to be a result of complications from the hysterectomy, and AEs exhibited no drug-related or dose-dependent pattern. The most common AEs reported by $\geq$4 patients in any group during the treatment period were headache, upper respiratory tract infections, nausea and vomiting, back pain or pain in extremities, procedural complications (intra- or post-operative haemorrhage) and abdominal pain. There were no clinically meaningful mean changes from baseline in hematology, coagulation, general chemistry, or urinalysis laboratory values. No clinically significant changes from baseline occurred for vital signs, physical findings, or ECG results.

Endometrial thickness measured by TVU increased slightly from screening to the final visit in the placebo (0.93mm) and asoprisnil 10mg (0.05mm) groups, respectively, and decreased by 4.5mm after treatment with asoprisnil 25mg. Pap test results revealed no clinically concerning changes in the asoprisnil groups. No patient had a symptomatic ovarian cyst during the study.

Table 3.5. Analysis of resistance and pulsatility indices (RI and PI)

Mean changes of RI and PI (impedance indices to determine uterine artery blood flow as determined by Colour Doppler imaging) from baseline to final visit in the three treatment groups (placebo, asoprisnil 10mg, asoprisnil 25mg)

[a] Change from baseline to final visit was analysed by a t-test with statistical significance at 0.05 level (*)

[b] From ANCOVA model for change from baseline to final visit with fixed effect of treatment and investigator and baseline mean RI / PI as a covariate;
statistical significance at 0.05 level using Hochberg's multiple comparison procedure ($^{\$}$)

NA = not applicable

	Treatment Group		
	Placebo (N=10) Mean ± SD	**Asoprisnil 10 mg (N=12) Mean ± SD**	**Asoprisnil 25 mg (N=11) Mean ± SD**
RI			
Baseline	0.73 ± 0.10	0.76 ± 0.09	0.71 ± 0.08
Final Visit	0.71 ± 0.17	0.75 ± 0.10	0.77 ± 0.08
Change – Baseline to Final Visit	-0.02 ± 0.13	-0.01 ± 0.06	0.06 ± 0.08
Within-group P-value[a] (change from baseline)	0.629	0.689	0.034*
Between groups P-value[b] Comparison vs placebo	NA	0.756	0.146
PI			
Baseline	1.69 ± 0.60	1.80 ± 0.72	1.52 ± 0.44
Final Visit	1.27 ± 0.33	1.81 ± 0.67	1.81 ± 0.48
Change – Baseline to Final Visit	-0.42 ± 0.42	0.01 ± 0.56	0.30 ± 0.54
Within-group P-value[a] (change from baseline)	0.012*	0.956	0.099
Between groups P-value[b] Comparison vs placebo	NA	0.019[$]	0.005[$]

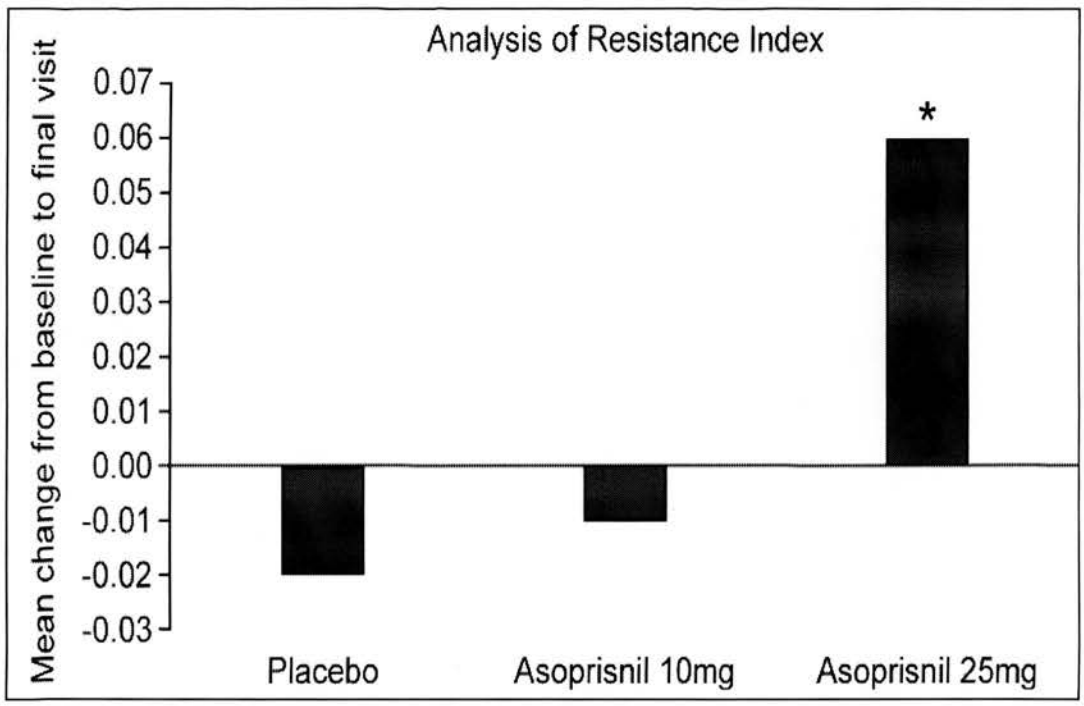


Figure 3.8. Analysis of Resistance Index (RI)

Mean changes of resistance index (RI; impedance index determined by colour Doppler imaging) from baseline to final visit in the three treatment groups (placebo, asoprisnil 10mg, asoprisnil 25mg); the significance of the change from baseline was determined using t-test at 0.05 level (*)

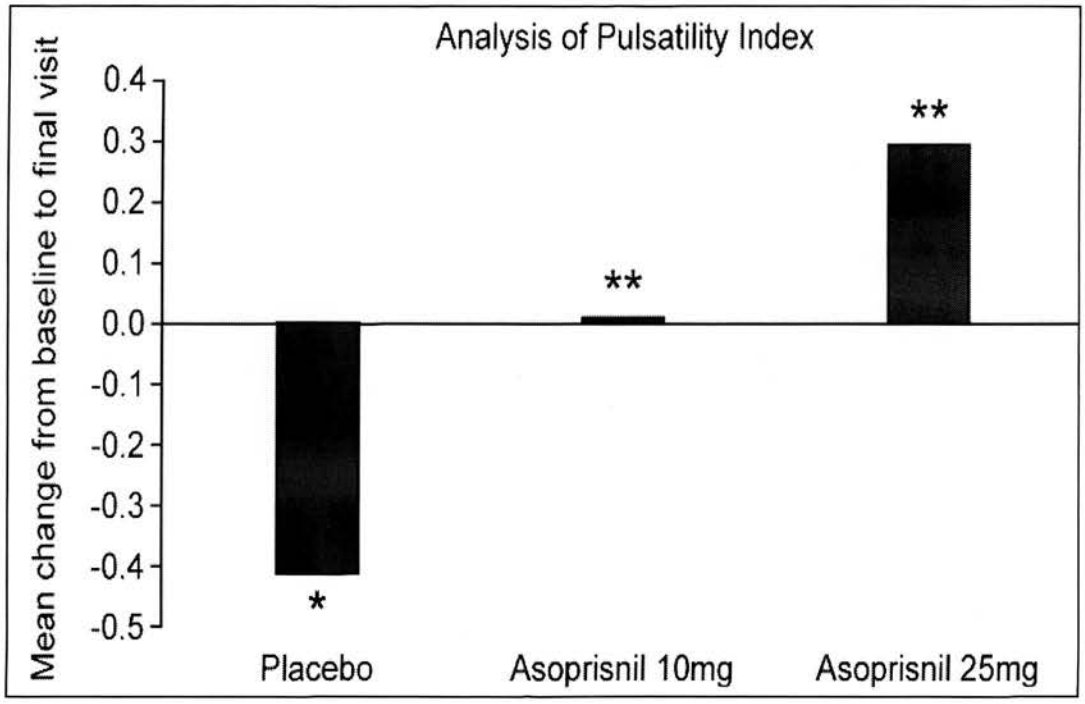


Figure 3.9. Analysis of pulsatility index (PI)

Mean changes of pulsatility index (PI; impedance index determined by colour Doppler imaging) from baseline to final visit in the three treatment groups (placebo, asoprisnil 10mg, asoprisnil 25mg); the significance of the change from baseline was determined using t-test at 0.05 level (*); the significance of the difference of change from baseline between placebo and asoprisnil groups was determined using Hochberg's multiple comparison procedure at 0.05 level (**)

	Treatment Group		
	Placebo (N=10) Mean ± SD	**Asoprisnil 10 mg (N=12) Mean ± SD**	**Asoprisnil 25 mg (N=11) Mean ± SD**
E1G (μmol/mol)			
Screening	11.5 ± 4.0	18.0 ± 7.2	15.0 ± 5.5
Weeks 1-4	12.0 ± 4.9	16.2 ± 4.5	11.6 ± 3.3
Weeks 5-8	13.3 ± 3.4	17.5 ± 6.6	12.5 ± 5.4
Weeks 9-12	12.0 ± 4.0	21.0 ± 4.4	10.7 ± 2.0
PdG (mmol/mol)			
Screening	0.42 ± 0.15	0.37 ± 0.15	0.39 ± 0.22
Weeks 1-4	0.39 ± 0.17	0.45 ± 0.26	0.17 ± 0.11
Weeks 5-8	0.50 ± 0.25	0.45 ± 0.33	0.23 ± 0.19
Weeks 9-12	0.45 ± 0.19	0.46 ± 0.27	0.15 ± 0.10

Table 3.6. Urinary E_1G and PdG levels

Mean levels of urinary oestrone glucuronide (E_1G) and pregnandiol glucuronide (PdG) collected twice weekly over four-week intervals during the screening cycle and during treatment with placebo, asoprisnil 10mg or asoprisnil 25mg; E_1G and PdG levels were measured using enzyme-linked immunoabsorbent assays and hormone concentrations corrected for creatinine excretion; levels are expressed as ratios of the creatinine concentration (E_1G in μmol/mol, PdG in mmol/mol)

Timepoint Algorithm	Treatment Group Placebo n/N (%)	 Asoprisnil 10 mg n/N (%)	 Asoprisnil 25 mg n/N (%)
Weeks 1 to 4			
Algorithm 1	1/10 (10)	4/12 (33)	8/10 (80)
Algorithm 2	2/10 (20)	4/12 (33)	9/10 (90)
Weeks 5 to 8			
Algorithm 1	0/10	4/12 (33)	7/10 (70)
Algorithm 2	0/10	4/12 (33)	7/10 (70)
Weeks 9 to 12			
Algorithm 1	0/10	2/11 (18)	7/10 (70)
Algorithm 2	2/10 (20)	2/11 (18)	8/10 (80)

Table 3.7. Number and percentage of patients with no evidence of luteal activity (NELA)

Numbers of patients in each treatment group with no evidence of luteal activity (NELA) over three consecutive time periods during the clinical study as evidenced by urinary pregnandiol glucuronide (PdG) levels according to two different algorithms as follows:

Algorithm 1 - defined evidence of luteal activity as a PdG concentration being at least 3 times as high as the minimum 3-concentration moving average during the past 28 days.

Algorithm 2 - same as Algorithm 1, with an additional requirement that PdG concentration must be >0.5 mmol/mol creatinine.

N = total number of subjects; n = number of subjects in subset

Two patients had ovarian cysts >3.5 cm during the treatment period: one patient on placebo underwent surgical removal of a simple cyst and another patient treated with 10mg asoprisnil had a complex cyst, which resolved spontaneously. Overall, treatment with asoprisnil for 12 weeks prior to hysterectomy was well tolerated.

3.4. Discussion

The objectives of this study were to assess the clinical effects of treatment with asoprisnil in women with uterine fibroids associated with symptoms severe enough to pose an indication for surgical intervention. Simultaneously, safety and tolerability were carefully evaluated. A rapid reduction in uterine bleeding, as evidenced by MP scores, was demonstrated as well as an improvement of other fibroid-related symptoms and quality of life measures. The effect of asoprisnil administered for three months on uterine artery blood flow was also investigated through ultrasonographic Doppler measurements of impedance (resistance and pulsatility indices) as a possible mechanism of action. Asoprisnil treatment was shown to be associated with a moderate decrease in uterine artery blood flow. Ovarian activity during treatment was determined using urinary PdG and E_1G levels. Whilst there was an apparent dose-dependent suppression of luteal activity, follicular activity continued or was only partially suppressed in most patients who were given asoprisnil.

3.4.1. Clinical symptoms

The profound effect of asoprisnil on menstrual bleeding pattern and improvement of fibroid-related symptoms demonstrated in this double-blind placebo-controlled study, is consistent with previous reports[142]. These findings emphasize the potential of asoprisnil for the medical management of uterine fibroids[139] and as a possible alternative to surgical intervention. The dramatic reduction in menstrual blood loss with asoprisnil has previously been described in women with[142] and without fibroids[137]. Asoprisnil has consistently suppressed menstruation and frequently achieved amenorrhoea in a dose-dependent manner.

In this study, the amount of blood loss was quantified with the aid of the menstrual pictogram (MP) rather than assessed semi-quantitatively. Consistent use of standardized sanitary products and the provision of visual analogues of the stained products on the pictogram allowed for quantification over three four-week periods during the study as well as the pre-treatment cycle, as previously described[167].

Statistically, menstrual blood loss during the third and final month of treatment was compared to the pre-treatment cycle in all treatment groups. Whilst there was a slight increase in the placebo group, the reductions in both asoprisnil groups were substantial (-154ml and -215ml, respectively after 10mg and 25mg asoprisnil). This represents a profound effect of asoprisnil on uterine bleeding, especially considering that the definition of heavy menstrual bleeding (HMB) has traditionally been a blood loss of over 80ml[147], and the reduction by treatment with asoprisnil 25mg is more than 2.5 times this amount. Even though statistically the final month of treatment was compared to baseline, substantial reductions were already apparent after the first month, highlighting not only a profound but also a rapid effect of asoprisnil on uterine bleeding. Not just the MP but also the record of the number of days with bleeding reflected a marked and dose-dependent reduction of bleeding by either dose of asoprisnil. The percentage of patients experiencing complete suppression of bleeding also appeared to be dose-related. Treatment with 25mg asoprisnil achieved suppression of uterine bleeding in 91% of patients, which is substantial particularly considering that some of these patients presented with menstrual blood loss of over 200ml according to their MP scores.

Managing HMB in the presence of uterine fibroids is often challenging and not infrequently an indication for hysterectomy. Due to the distortion and enlargement of the uterine cavity, management options such as the levonorgestrel-releasing intrauterine system (LNG-IUS) or endometrial ablative techniques may not be appropriate and have limited success rates[152]. In this and previous studies[138,142], asoprisnil suppressed menstrual bleeding independent of the size and location of uterine fibroids. The high rates of amenorrhoea achieved with the 25mg dose of asoprisnil highlight its potential role for the conservative management of women with heavy bleeding and uterine fibroids.

In addition to the effect on uterine bleeding, the symptom of dysmenorrhoea was significantly improved after treatment with either dose of asoprisnil compared to the placebo group. Most likely this is due to the suppression of menstruation. Other fibroid-related symptoms (bloating, pelvic pressure, urinary symptoms) did also improve even though the difference to the placebo group was not statistically significant. This trend suggests that treatment with asoprisnil is beneficial for a variety of symptoms, which may be associated with uterine fibroids. The UFS-QOL focussed specifically on the impact of these symptoms on quality of life. In this study, treatment with asoprisnil achieved statistically significantly greater improvements in most of the disease-specific UFS-QOL domains than placebo. As previously described, responses to the UFS-QOL questionnaire were grouped into

subscales of concern, activities, energy / mood, control, self-consciousness, sexual function and symptom severity[169]. The improvement from baseline to final visit was reported with both doses of asoprisnil and on all subscales. The difference to placebo was statistically significant in the subscales of concern, activities, control and self-consciousness and for the total UFS-QOL score. The difference in reduction of symptom severity was only statistically significant after 25mg asoprisnil. In addition to its effects on measurable parameters such as menstrual blood loss and fibroid volumes, asoprisnil therefore appears to also have a significant impact on patients' perception of their symptoms and on their quality of life. All patients in this study had presented with symptoms, which were significant enough for them to consent to major surgery for benign disease. In this cohort of patients with debilitating symptoms, 12 weeks of treatment with 25mg asoprisnil resulted in amenorrhoea in 91% of cases and 100% improvement in dysmenorrhoea. Menstrual dysfunction is the most common complaint associated with uterine fibroids and so not surprisingly, asoprisnil also made a statistically significant difference to the quality of life of these women whose only option for improvement was to resort to hysterectomy.

3.4.2. Uterine artery blood flow and size of fibroids

The results of previous clinical studies have suggested that uterine artery blood flow is an important variable influencing the growth of uterine fibroids. Uterine artery blood flow has been shown to increase in patients with uterine fibroids[176]. Conversely, there is evidence that pharmacological agents such GnRH analogues[177] and danazol[178] reduce uterine artery blood flow as well as fibroid volumes. The same combined effects have been demonstrated in a study with the progesterone antagonist mifepristone[156]. Hence, in this study, the effect of asoprisnil on uterine artery blood flow was assessed to investigate a possible mechanism of action in the presence of uterine fibroids. With measurements of impedance in the uterine arteries via the resistance and pulsatility indices (RI and PI), a statistically significant effect of both doses of asoprisnil on PI was demonstrated compared to placebo. There was also a trend towards increased RI. Together, these findings are suggestive of a moderate inhibitory effect of asoprisnil on uterine artery blood flow.

The presumably causative association between reduced uterine artery blood flow and decreasing volumes of uterine fibroids is the rationale behind treatment of symptomatic fibroids by uterine artery occlusion techniques such as uterine artery embolization (UAE)[152,179]. It has been demonstrated that UAE significantly reduces the size of fibroids as well as the overall uterine volumes. This was associated with a

substantial improvement of symptoms such as heavy menstrual bleeding, dysmenorrhoea, pressure symptoms and also urinary symptoms and sciatica[154]. The impact of UAE on quality of life has been shown to be comparable to surgical intervention for uterine fibroids[152]. There is evidence that reduced uterine artery blood flow may also be achieved pharmacologically, for example with GnRH analogues[177]. GnRH analogues are the mainstay of current medical management of uterine fibroids and are known to be effective in reducing volumes of the fibroid uterus[86,180]. The finding of intravaginal misoprostol reducing uterine artery blood flow has led to the suggestion it could be used in patients with fibroids even though this effect may be secondary to uterotonic action[181]. Danazol therapy for six weeks, whilst improving symptoms of dysfunctional bleeding, caused increased uterine artery impedance resulting in reduced blood flow[178]. The present study shows that the SPRM asoprisnil has similar properties even though it remains to be investigated how exactly the effect on the uterine artery blood flow is exerted. It appears that the effect of asoprisnil on decreasing fibroid volumes may be at least partially mediated by reduced uterine artery blood flow.

Twelve weeks of treatment with either dose of asoprisnil resulted in a reduction of the sonographically measured volume of the largest fibroid, whilst this increased in the placebo group. This is consistent with previous reports[168], although in this study, the difference between groups was not statistically significant. The reduced size was accompanied by decreased mitotic activity in leiomyoma tissue and a more common appearance of degenerative changes in patients receiving asoprisnil[135]. There appear to be a multitude of direct and indirect mechanisms involved in the asoprisnil-related reduction of fibroid volumes. Decreased uterine artery blood flow is likely to contribute, even though it is probably not the primary mechanism.

With the recognition that progesterone stimulates fibroid development and growth[84,85], a compound with partial antagonist effects on progesterone target tissues would be expected to have antiproliferative properties. Evidence from in vitro studies is growing regarding suppressed proliferation and induction of apoptosis in cultured leiomyoma cells exposed to asoprisnil, whilst no such effect is seen in myometrial cells[182,183]. Treatment of cultured leiomyoma cells with asoprisnil has been shown to result in down-regulation of growth factors (epidermal growth factor (EGF), insulin-like growth factor-I (IGF-1) and transforming growth factor β3 (TGF β3) and their receptors (pEGF-R, IGF-1Rα, pTGFβ-RII). Asoprisnil also reduced the number of viable leiomyoma cells, which is indicative of an antiproliferative effect. The same treatment of myometrial cells did affect neither growth factor expression nor cell proliferation[184]. In vitro studies have not only demonstrated the suppressive effect of

asoprisnil on proliferation of leiomyoma cells but also a stimulating effect on apoptosis. The tumour necrosis factor-related apoptosis-inducing (TRAIL)-mediated signalling pathway was up-regulated by asoprisnil in leiomyoma cells, whilst the inhibitor of apoptosis protein (Bcl-2) was down-regulated. These effects were reversible by simultaneous treatment with progesterone and not observed at all in myometrial cells[182,185].

Overall, the results of these studies suggest that asoprisnil has selective antiproliferative effects on leiomyoma cells via down-regulation of growth factors and their receptors as well as induction of apoptosis[183] mediated by the progesterone receptor (PR). They indicate that asoprisnil targets uterine fibroids directly and selectively, which is in contrast to the mode of action of GnRH analogues. GnRH analogues down-regulate ovarian secretion of oestrogen and progesterone via the pituitary gland and achieve a reduction in total uterine volume[86], whilst asoprisnil specifically decreases the size of fibroids.

3.4.3. Ovarian activity

In contrast to GnRH analogues, the present and previous studies have shown clinical and antiproliferative effects of asoprisnil to occur in the presence of circulating follicular phase oestrogen concentrations[168]. This implicates that side effects due to hypo-oestrogenism would not be expected and have not been described. In this study, follicular and luteal ovarian activity were investigated separately and in some detail by assessing urinary PdG and E_1G twice weekly throughout the treatment period. Luteal activity was suppressed by treatment with asoprisnil, and this effect appeared to be dose-dependent. Follicular activity was evaluated by comparing urinary E_1G levels during treatment to levels prior to dosing. Suppression of follicular activity by asoprisnil was also dose-dependent but less consistent than the effect on luteal activity. Most patients experienced continued or only partially suppressed follicular activity whilst on treatment with asoprisnil. These findings indicate that asoprisnil inhibits luteinization in the majority of women at the higher dose (25 mg), whilst follicular activity and therefore ovarian oestrogen secretion is maintained. Previous study have consistently reported asoprisnil to exert its clinical effects including suppression of menstruation in the presence of follicular phase oestrogen concentrations[137,168]. The fact that asoprisnil achieves its dramatic effects on symptomatic uterine fibroids whilst physiological oestrogen concentrations are maintained, emphasizes its clinical potential for this group of patients. The risk of hypo-oestrogenism is the main limiting factor for the long-term use of GnRH

analogues[186], and yet currently they are often the only medical management option for control of symptoms due to uterine fibroids such as heavy bleeding and / or pelvic pressure.

3.4.4. Safety parameters

In this study, treatment with asoprisnil was well tolerated. There were no premature terminations during the treatment phase, and all patients completed the study with good compliance. The majority of adverse events (AEs) were mild or moderate in severity. The timing of AEs indicated that many were a result of the planned hysterectomy or complications from that surgery. There were no clinically meaningful changes from baseline in haematology, coagulation, general chemistry, or urinalysis laboratory values. These findings are consistent with previous studies showing asoprisnil to have a favourable safety and tolerability profile[137,168].
There was no increase in the sonographically measured endometrial thickness and indeed a decrease after 12 weeks of treatment with asoprisnil 25mg. This trend for decreased endometrial thickness was also found, when histological sections of the endometrium were measured by eyepiece micrometer[135]. These findings are consistent with an antiproliferative effect of the SPRM asoprisnil on human endometrium as previously observed in animal models[139]. This aspect will be further explored in the next two chapters.

3.4.5. Limitations of the study

The effects of asoprisnil on clinical symptoms associated with uterine fibroids appears to be profound and rapid, as they were apparent after only one month of treatment. However, this study is not able to demonstrate whether this effect will be maintained long term, as the study period of 12 weeks is relatively short. It also appears that most of the symptom relief is related to the suppression of uterine bleeding. As heavy menstrual bleeding was the predominant symptom in most patients in this study, it is difficult to independently assess any effect of asoprisnil on other fibroid-related complaints. It would have been interesting to specifically ask every patient prior to hysterectomy, whether they would have chosen to avoid surgery, had asoprisnil remained available as a management option. As it stands, the responses to the UFS-QOL may suggest their views on this, but other factors not included in the study may also influence patients' preference for a certain treatment modality.

This study suggests that asoprisnil reduces uterine artery blood flow, which may represent a possible mechanism of action. It is important to bear in mind the possible inter- and intra-observer variability of colour Doppler imaging. Every effort was made in this study to limit the opportunites for error by specifying the menstrual cycle phase for obtaining measurements prior to commencing the study drug and by ensuring that the majority of investigations were carried out by one specialized radiologist per study site. However, as there were four study sites for 33 patients, the possibility of inter-observer errors needs to be considered.

The effects of asoprisnil on ovarian activity in this study were investigated by measuring urinary PdG and E_1G levels twice weekly, To assess the effect on ovulation, serial ultrasound assessments of the dominant follicle and more frequent measurements of the ovarian and pituitary hormone levels would be required. Urinary PdG levels indicate the presence of progesterone as a result of either ovulation or a luteinized unruptured follicle. In this study, it is not possible to differentiate whether ovulation has occurred or not.

3.5. Conclusion

In patients with symptomatic uterine fibroids scheduled for hysterectomy, asoprisnil taken for 12 weeks substantially reduced blood loss as evidenced semi-quantitatively by menstrual pictogram evaluation. It also improved other symptoms and quality of life measures, as indicated by responses to the LSAQ and the UFS-QOL. Asoprisnil was shown to moderately reduce uterine artery blood flow demonstrated by altered resistance and pulsatility indices. This effect may contribute to fibroid volume reduction, as demonstrated in this study, even though it is unlikely to be the primary mechanism. All these effects were seen without significant alterations of serum oestrogen levels. 10 mg and 25 mg doses of asoprisnil were safe and effective when administered daily for a 12-week period. Further studies are needed to determine the safety and efficacy profile of asoprisnil when administered beyond 12 weeks.

CHAPTER 4

ENDOMETRIAL MORPHOLOGY

The data presented in this chapter have previously been peer-reviewed and published in the manuscript:

Williams ARW, Critchley HOD, Osei J*, Ingamells S, Cameron IT, Han C and Chwalisz K
The effects of the selective progesterone receptor modulator asoprisnil on the morphology of uterine tissues after 3 months treatment in patients with symptomatic uterine leiomyomata.
Human Reproduction 2007; 22(6): 1696-704
(* At the time of this publication, the author's surname was Osei)

The tables in this chapter have been adapted from the same manuscript.
The author was personally involved in the recruitment and clinical care of the patients in Edinburgh and the tissue collection at the time of hysterectomy. Grateful acknowledgement is due to Dr Alistair Williams for performing the histological evaluation and analysis on the endometrial specimen.

4.1. Introduction

Endometrial morphology is recognized to change considerably according to the different phases of the menstrual cycle under the influence of the ovarian steroid hormones. These changes are so distinct that the cycle phases are classified into proliferative and secretory by the endometrial appearances. Follicular ovarian activity results in increased secretion of oestrogen (E), which promotes endometrial proliferation with frequent mitoses, particularly in the functional layer. Following ovulation, luteal ovarian activity subsequently raises progesterone (P) levels transforming the endometrium into a secretory state required for successful implantation. Glandular as well as stromal compartments exhibit characteristic changes dependent upon the exposure to ovarian steroid hormones. The typical endometrial appearances have been correlated to the cycle stage and used to determine the phase of the cycle during which an endometrial specimen was obtained[6,7].
Quite expectedly, endometrial morphology may be further modified by exogenous steroid hormones or by conditions altering steroid activity in the endometrium. Notably, exposure to unopposed E acts as a protracted proliferative stimulus and is well known to result in endometrial hyperplasia with an increased risk of endometrial cancer[187-189]. The mitogenic effect of E may be unopposed due to persistent

anovulation (i.e. with polycystic ovarian syndrome or in the perimenopause) or with hormone replacement therapy. Prolonged and continuous administration of progestins is currently the basis for medical management of many benign gynaecological conditions as well as most forms of hormonal contraception. The highest endometrial levels of progestin are achieved with delivery via an intrauterine system such as the levonorgestrel-releasing intrauterine system (LNG-IUS). Endometrial appearances in LNG-IUS users have been studied and described as glandular atrophy, extensive stromal decidualization with altered blood vessel integrity[190]. These morphological changes appear to be related to the clinical effects of suppressed uterine bleeding even though the common occurrence of breakthrough bleeding has not been sufficiently explained.

Progesterone receptor modulators (PRMs) have mixed and partial progesterone receptor (PR) agonist and antagonist properties. Mifepristone was the first developed compound within this category and has mostly PR antagonistic activity. This may raise the concern of causing endometrial changes as seen with exposure to unopposed oestrogen. However, very soon after discovery of mifepristone, its endometrial antiproliferative effect was described in non-human primates[191]. This paradoxical observation was termed the functional non-competitive anti-oestrogenic effect, as it occurred in the presence of physiological oestrogen levels and was specific to the endometrium. The same phenomenon has since been observed with other PRMs in non-human primates and women[123]. In cynomolgus macaques, the endometrial antiproliferative effect was characterized by reduced mitotic activity, decreased thickness and wet weight in conjunction with glandular apoptosis, stromal atrophy and stromal compaction. Additionally, the spiral arteries showed hyalinizing degeneration with a narrowed lumen[123]. Similarly in humans, the endometrial appearances following administration of low dose (2-5mg) mifepristone have been described as suppressed glandular proliferation with reduced mitotic indices and increased stromal density[124,192].

Other features seen in endometrium exposed to mifepristone and other PRMs have previously been described as simple hyperplasia[162]. This diagnosis was made mainly on the basis of prominent cystically dilated glands reminiscent of the appearances of the disordered proliferative endometrium due to the effect of unopposed E. Further detailed examination revealed however, that unlike the proliferative lining of the glands seen with hyperplasia, the glands in endometrium after administration of PRMs were only weakly mitotic and sometimes secretory. It was realized that PRMs cause changes to the endometrial morphology, which are unique to this class of compounds[136,193]. These appearances are not amenable to conventional assessment

using the Noyes criteria[6] and have since been characterized as non-physiologic secretory effects[137].

It has not been fully explained by which mechanism PRMs exert their effects on endometrial morphology and on clinical symptoms. It has been hypothesized that the endometrium is directly targeted, as most effects are evident irrespective of continuing follicular and luteal ovarian activity. The specific endometrial changes, particularly within the stroma and the vasculature, may mediate the clinical suppression of uterine bleeding[137]. Hence, the detailed study of endometrial morphology after administration of PRMs is most important for two reasons. It may serve to further elucidate the exact mechanism of action of these compounds. It is also vital in order to ensure the safety of long-term administration.

In this study, detailed histological assessment was carried out in full thickness endometrial samples from hysterectomy specimen after administration of 10 or 25mg asoprisnil for 12 weeks prior to surgery. Unlike samples obtained with a Pipelle, full thickness specimens include the basal endometrial layer. These full thickness endometrial samples retain their cellular architecture and thus provided the opportunity to evaluate the effects of asoprisnil in all layers of the endometrium.

4.2. Materials & Methods

4.2.1. Tissue collection

Endometrial biopsies were collected from all patients with a Pipelle endometrial sampler within 3 months prior to commencement of the study. It was an absolute requirement for continuation in the study for this biopsy to be satisfactory for assessment and normal.

Endometrial samples from the hysterectomy specimen were obtained as described in section 2.2.1 and a previous publication[135]. For morphological assessment, three blocks each were taken from the uterine fundus, mid-corpus and isthmus, respectively. These were processed by routine methods. 3μm haematoxylin-eosin stained sections were prepared by microtomy and mounted on glass slides. A section was considered unsuitable for assessment, if a fibroid was present within 10mm of the endometrium.

Samples from myometrium and fibroids were also obtained from each patient. In some cases, non-endometrial tissue (ovaries, Fallopian tubes, cervix) was collected at hysterectomy and submitted for histological examination.

Morphology	Category
Variants of normal cycle	Simple tubules - minimal undulation
	Simple tubules - undulation as in normal proliferative phase
	Tubules - undulation as in early secretory phase
	Glands with morphology of mid or late secretory phase
	Mixed (<80% show one pattern)
Cystic dilatation	None
	<10% of glands
	10–25% of glands
	>25% of glands
Folding patterns	No abnormal folding
	1-2 glands
	3-6 glands
	>6 glands
Gland-stroma ratio	<1:5
	1:5 – 1:1
	>1:1 and <3:1
	≥3:1
Cell height	Flattened – height < width
	Cuboidal – height = width
	Columnar – height > width
Secretory effects	No cytoplasmic vacuolation
	Sub- and/or supranuclear vacuolation
	Apical secretion +/- cytoplasmic vacuolation
	Intraluminal secretion
Nuclear stratification	None
	<10%
	10–50%
	>50%
Nuclear atypia	Absent
	Present in 1-2 glands
	Present in >2 glands, up to 50%
	Present in >50% of glands

Table 4.1. Morphological categories of endometrial glandular histology[135]

Morphology	Category	
Cellularity[a]	Atrophic[b]	Compact
		Variable oedema
		Oedematous
	Non-atrophic	Compact
		Variable oedema
		Oedematous
Decidual change	None	
	Around vessels only	
	Around vessels, extending into stroma (non confluent)	
	Confluent	
Thin walled vessels	No dilatation	
	Dilatation in <50%	
	Dilatation in ≥50%	
Mural muscularization of vessels	None	
	Normal in <50%	
	Normal in ≥50%	
	Presence of abnormally muscularized arterial vessels[c]	
Aggregations of vessels[d]	None	
	1-2	
	>2	
[a] Non-decidualized stroma only		
[b] Atrophic defined as nucleus / cytoplasm ratio ≥1		
[c] Abnormal arterial vessels similar to those seen in stroma of endometrial polyps		
[d] Number of vessel aggregates per slide		

Table 4.2. Morphological categories of endometrial stromal histology[135]

4.2.2. Histological assessment

The Pipelle endometrial biopsies obtained prior to treatment were submitted to routine pathological evaluation. The "Dictionary of Endometrial Biopsy Diagnoses for Clinical Trials with SPRMs" was used to classify the endometrial appearances of the hysterectomy samples in this study. This was developed by TAP Pharmaceuticals Inc (Lake Forest, IL, USA), Diagnostic Cytology Laboratories (Indianapolis, IN, USA) and a group of expert gynaecological pathologists, when it became evident that the unique endometrial changes encountered following exposure to PRMs were not assessable by the conventional criteria of endometrial dating[6]. This system is based on the currently used criteria of the normal menstrual cycle as described by Noyes[6] and in Blaustein's Pathology of the Female Genital Tract[143], but includes further categories, which are considered characteristic of the PRM effects.

A single expert gynaecological pathologist evaluated all sections whilst blinded to treatment groups. The maximum single endometrial thickness on each section was measured using an eyepiece micrometer. Overall endometrial appearances were categorized according to the classification system as above. Individual histological characteristics of glandular, stromal and vascular compartments were analysed using further descriptive subcategories (Tables 4.1 & 4.2).

Any histological changes or abnormalities in the myometrium and any degenerative effects within the fibroids were noted. The samples from outwith the uterus were screened for any changes within their vascular beds to assess whether the altered appearances in the endometrium were site-specific.

4.2.3. Assessment of mitotic activity

The number of unequivocal mitotic figures present per 10 high-power microscope fields of endometrium (Olympus BX51, x40 objective, field diameter 0.55mm) were counted separately in glandular and stromal compartments and expressed as mitoses per unit area. First, the area with the greatest density of mitoses was identified by microscopic assessment for counting to commence. Thereafter, a minimum of 10 non-overlapping fields was randomly selected for counting.

4.2.4. Data analysis

The numbers and percentages of patients in each diagnostic category were summarized for each treatment group and for endometrial samples from each of the

intrauterine locations (fundus, mid-corpus, isthmus). The same procedure was performed for the more detailed evaluation of the morphological features in glands and stroma. No statistical analysis was performed for these descriptive assessments.
For each treatment group and each of the three sampling locations, mean and standard deviation were calculated for the endometrial thickness. The t-test was applied to compare the thickness between placebo and combined asoprisnil groups for each location separately. Hochberg's multiple comparison procedure was used to adjust for the multiplicity resulting from the three sampling locations.

4.3. Results

4.3.1. Histological assessment

All Pipelle endometrial biopsies obtained at baseline were confirmed normal and classified as inactive (10%), proliferative (28%), secretory (55%) or menstrual (7%). Samples from all three sampling locations (fundus, mid-corpus, isthmus) were available for histological assessment in 29 cases. Within the placebo group, normal secretory appearances were seen in 71% of available samples from each of the three locations. The majority of samples from either of the asoprisnil groups (50-73%) were assigned to the non-physiologic secretory category reflecting the PRM-specific endometrial effect. Administration of 25mg asoprisnil resulted in assignment to this category in 73% of samples from both fundus and mid-corpus. Non-physiologic secretory endometrial appearances were seen in only one placebo-treated case in the samples from fundus and mid-corpus (Table 4.3.).

4.3.2. General endometrial morphology following exposure to asoprisnil

The non-physiologic secretory effect commonly seen in endometrium after administration of asoprisnil, is characterized by a combination of morphological features in glands, stroma and vasculature. All tissue compartments need to be assessed in order to assign a sample to this category, as none of the individual features on their own are specific for exposure to PRMs.
Generally, the endometrium from asoprisnil-treated patients tended to be too thin to distinguish functional and basal layers (Figure 4.1.A). The morphological appearances associated with exposure to asoprisnil were similar near the surface and near the myometrial interface, hence presumably occurred in functional and basal layers alike.

		Diagnostic category				
Treatment	**Site in uterus**	Inactive n (%)	Normal proliferative n (%)	Normal secretory n (%)	Non-physiologic secretory n (%)	Un-satisfactory n (%)
Placebo	Fundus n = 7	0 (0)	1 (14)	**5 (71)**	1 (14)	0 (0)
	Corpus n = 7	0 (0)	1 (14)	**5 (71)**	1 (14)	0 (0)
	Isthmus n = 7	0 (0)	1 (14)	**5 (71)**	0 (0)	1 (14)
Asoprisnil 10mg	Fundus n = 10	0 (0)	2 (20)	1 (10)	**7 (70)**	0 (0)
	Corpus n = 10	1 (10)	2 (20)	2 (20)	**5 (50)**	0 (0)
	Isthmus n = 10	1 (10)	3 (30)	0 (0)	**5 (50)**	1 (10)
Asoprisnil 25mg	Fundus n = 11	1 (9)	1 (9)	1 (9)	**8 (73)**	0 (0)
	Corpus n = 11	1 (9)	0 (0)	1 (9)	**8 (73)**	1 (9)
	Isthmus n = 10	3 (30)	1 (10)	0 (0)	**5 (50)**	1 (10)

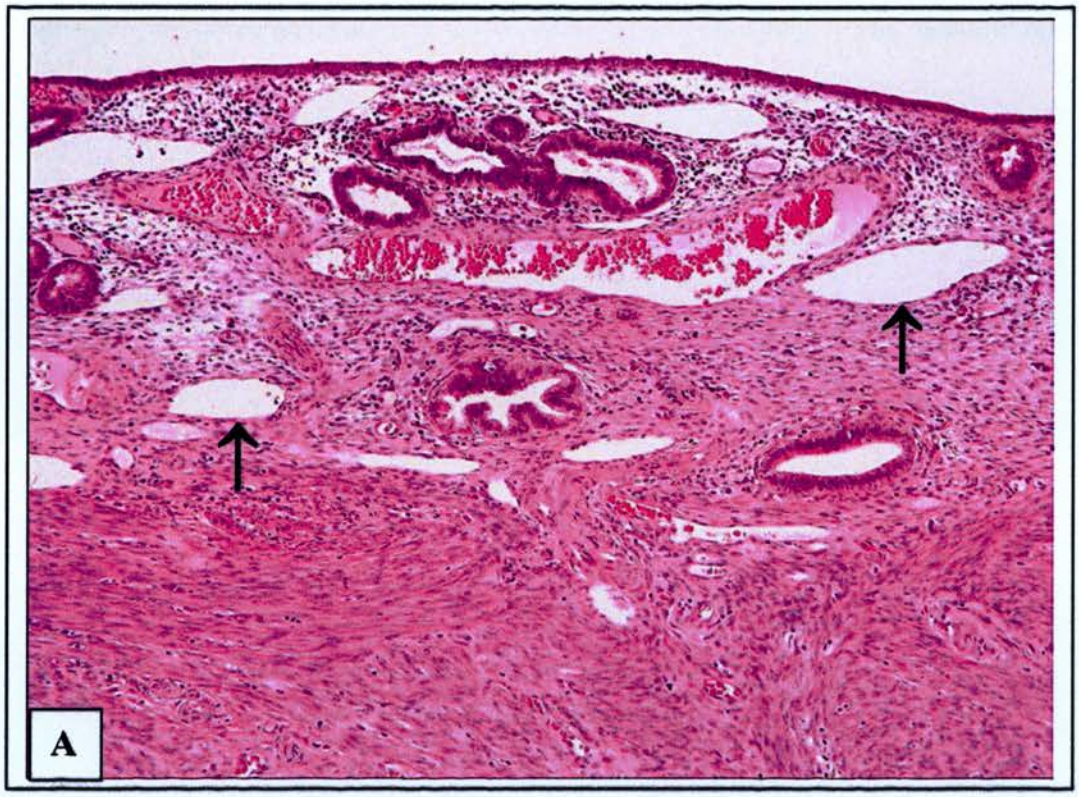
Table 4.3. Overall diagnostic category assigned to endometrial specimens[135]

Figure 4.1. Endometrial vascular morphology following exposure to asoprisnil

A Thin endometrium with dilated thin-walled vessels (arrows)

B Clusters of thick-walled muscularized spiral arterioles within the endometrial stroma

(Reproduced with permission from Dr Alistair Williams, Department of Pathology, University of Edinburgh)


A

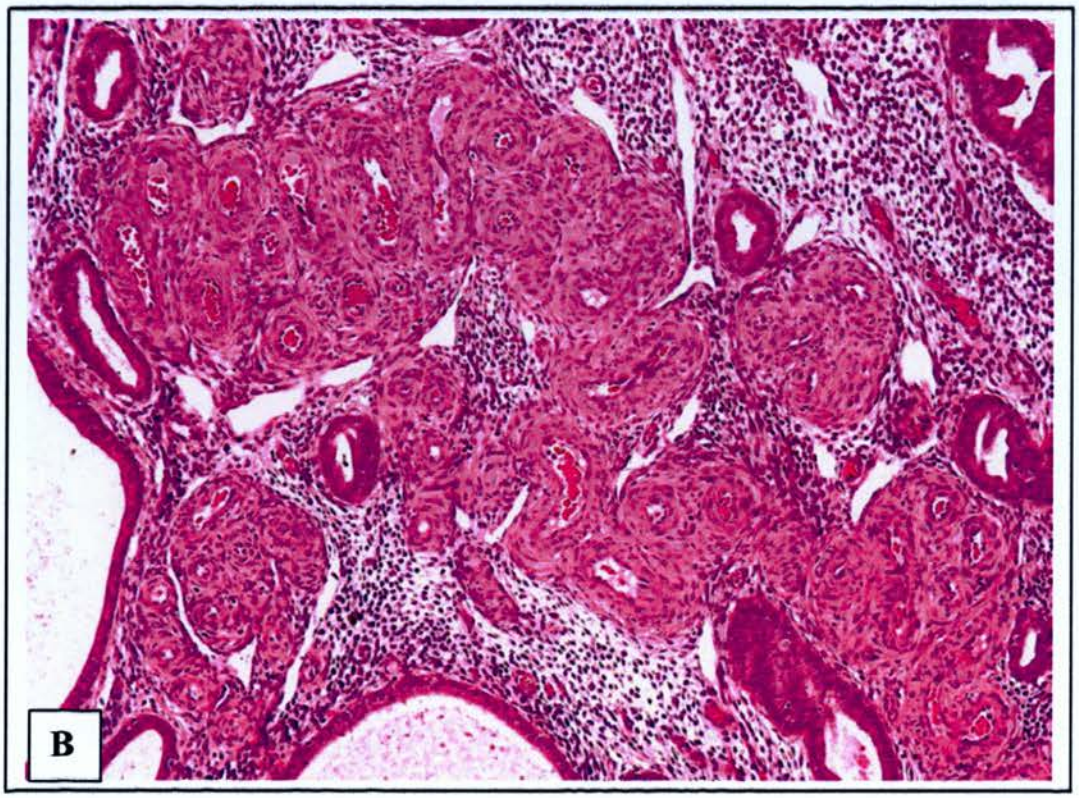
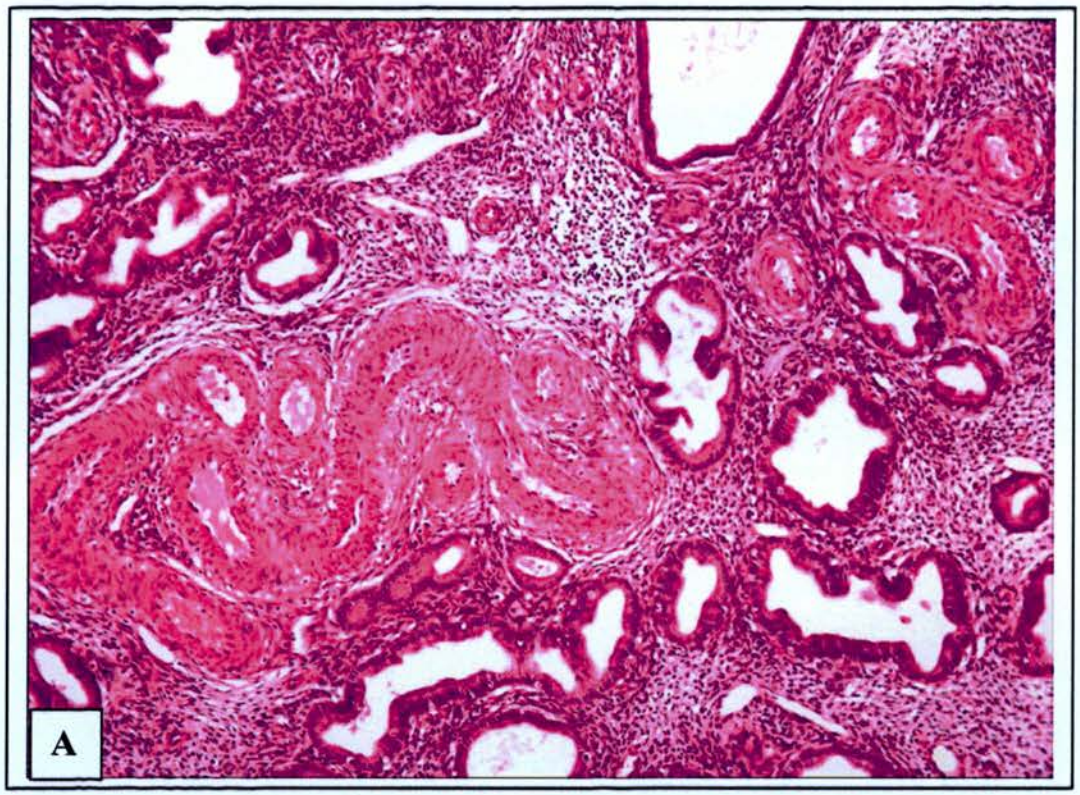

B

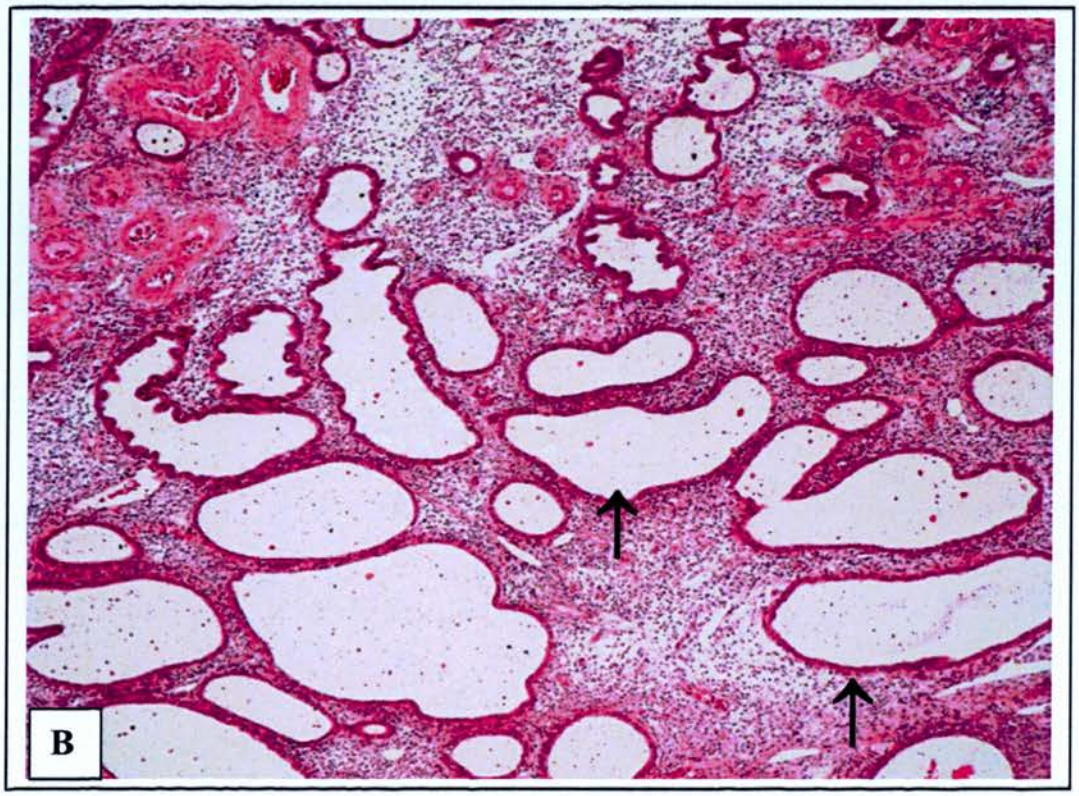
Figure 4.2. Endometrial glandular morphology following exposure to asoprisnil

A Non-physiologic secretory appearance of endometrial glands with tortuous or serpentine profiles similar to the normal mid-secretory phase

B Cystic glandular dilatation is commonly seen with asoprisnil (arrows)

(Reproduced with permission from Dr Alistair Williams, Department of Pathology, University of Edinburgh)


A


B

Most striking and most consistently identified after asoprisnil administration were the features of the endometrial vasculature. The endometrial stroma commonly displayed clusters of thick-walled muscularized vessels similar to the vascular appearances in benign endometrial polyps (Figure 4.1.B). Thin-walled vessel aggregates were also frequently identified throughout the endometrium (Figure 4.1.A). Whilst none of the clusters of thick-walled muscularized vessels were found in endometrial samples from placebo-treated patients, dilated thin-walled vessels did occur occasionally but much less commonly than in the asoprisnil-treated groups.
The glandular pattern was reminiscent of secretory differentiation with serpentine or tortuous profiles and columnar epithelium showing few or no mitotic figures (Figure 4.2.A). Despite the secretory appearances, evidence of active secretion such as cytoplasmic vacuolation was very rarely observed. Cystic glandular dilatation was common but without any abnormal folding patterns and without increasing the gland-stroma ratio above normal (Figure 4.2.B). There was a tendency for increased stromal compactness but no evidence of decidualization.

4.3.3. Endometrial thickness

The measurements of the single layer endometrial thickness as obtained with the eyepiece micrometer from the histological sections of the hysterectomy specimen are shown in Table 4.4. The ranges over the different intrauterine locations were 3.1 – 4.1mm single layer thickness in the placebo group, 2.1 – 2.6mm with 10mg asoprisnil and 1.3 – 2.8mm with 25mg asoprisnil. Even though there appeared to be a trend for asoprisnil to decrease endometrial thickness, the differences between placebo and the combined asoprisnil groups were not statistically significant.

4.3.4. Mitotic activity

The numbers of endometrial sections from uterine fundus, mid-corpus and isthmus showing 0, 1, 2-5 or >5 mitotic figures per 10 high-power fields (HPFs) within the glands or stroma are shown in Table 4.5. The use of samples from placebo-treated patients for comparison is limited due to the variable cycle phases. Most patients in the placebo group underwent hysterectomy during the secretory phase of their menstrual cycle, when mitoses would be expected to be infrequent or absent. However, it is notable that following administration of 25mg asoprisnil, no mitotic activity was identified in endometrial glands except in one sample.

Treatment	Site in uterus	Mean endometrial thickness (in mm)	Standard deviation (SD)
Placebo	Fundus n = 7	**3.60**	1.988
	Corpus n = 7	**4.06**	2.018
	Isthmus n = 6	**3.05**	1.907
Asoprisnil 10mg	Fundus n = 10	**2.62**	1.307
	Corpus n = 10	**2.14**	0.977
	Isthmus n = 9	**2.26**	1.840
Asoprisnil 25mg	Fundus n = 11	**2.79**	1.718
	Corpus n = 11	**2.22**	1.098
	Isthmus n = 10	**1.27**	0.625

Table 4.4. Mean endometrial thickness[135]

Measurements of single layer endometrial thickness are taken on the histological sections from the hysterectomy specimen with the eyepiece micrometer;
the apparent trend of reduced endometrial thickness with asoprisnil treatment is not statistically significant at 0.05 level

Treatment	Site in uterus	Number of mitoses per 10 high-power fields - n (%)									
		Glands					Stroma				
		0	1	2-5	>5	US	0	1	2-5	>5	US
Placebo	Fundus n = 7	5 (71)	0	1 (14)	1 (14)	0	3 (43)	2 (29)	0	2 (29)	0
	Corpus n = 7	5 (71)	0	0	2 (29)	0	2 (29)	1 (14)	4 (57)	0	0
	Isthmus n = 7	4 (57)	0	1 (14)	1 (14)	1 (14)	3 (43)	0	3 (43)	0	1 (14)
Asoprisnil 10mg	Fundus n = 10	5 (50)	3 (30)	1 (10)	1 (10)	0	6 (60)	1 (10)	3 (30)	0	0
	Corpus n = 10	5 (50)	2 (20)	2 (20)	1 (10)	0	7 (70)	3 (30)	0	0	0
	Isthmus n = 10	5 (50)	2 (20)	0	1 (10)	2 (20)	6 (60)	2 (20)	0	0	2 (20)
Asoprisnil 25mg	Fundus n = 11	9 (82)	1 (9)	0	0	1 (9)	8 (73)	1 (9)	1 (9)	0	1 (9)
	Corpus n = 11	10 (91)	0	0	0	1 (9)	9 (82)	0	1 (9)	0	1 (9)
	Isthmus n = 9	9 (100)	0	0	0	0	9 (100)	0	0	0	0

Table 4.5. Mitotic activity[135]

Number of samples with 0, 1, 2-5 or >5 mitotic figures per 10 high-power fields (HPFs);
US denotes section unsatisfactory for assessment of mitotic figures

Treatment	Site in uterus	Aggregates of thin-walled vessels n (%)	Thick-walled muscularized vessels n (%)
Placebo	Fundus n = 7	2 (29)	0
	Corpus n = 7	1 (14)	0
	Isthmus n = 7	0	0
Asoprisnil 10mg	Fundus n = 10	3 (30)	2 (20)
	Corpus n = 10	5 (50)	3 (30)
	Isthmus n = 10	2 (20)	1 (10)
Asoprisnil 25mg	Fundus n = 11	5 (45)	5 (45)
	Corpus n = 11	5 (45)	5 (45)
	Isthmus n = 10	4 (40)	3 (30)

Table 4.6. Endometrial stromal vessels[135]

Number of endometrial samples showing aggregates of thin-walled vessels and thick-walled muscularized vessels

In the stroma, mitotic figures were found in three samples, whilst they were absent from 73%, 82% and 100% of specimens from fundus, mid-corpus and isthmus, respectively. Treatment with 10mg asoprisnil resulted in one mitosis or less per 10 HPFs in at least 70% of samples from both glands and stroma.

4.3.5. Specific morphology of different tissue compartments

4.3.5.1. Endometrial glands

The gland architecture in samples from the asoprisnil-treated groups varied from simple tubules with minimal undulation to tortuous appearances reminiscent of the normal mid- or late secretory phase. Following the higher asoprisnil dose of 25mg, the simple tubular pattern was more commonly observed in samples from the uterine isthmus (40%) than in fundus or mid-corpus (9%). Despite the architecture mostly resembling the secretory phase, cytoplasmic secretory vacuolation was absent from the glands in all patients taking 25mg asoprisnil and infrequent (up to 30%) with the lower 10mg dose. The predominant epithelial cell type in endometrial glands from all samples consisted of tall columnar cells with a degree of nuclear stratification that did not vary significantly between treatment groups.

Some degree of cystic gland dilatation was evident in all uterine locations after exposure to 25mg asoprisnil but particularly in the isthmus (80% compared to 29% in the placebo group). No cytological atypia in epithelial cell nuclei was identified in any patient. There was no difference in the frequency of glandular morphological abnormalities other than cystic dilatation between placebo- and asoprisnil-treated patients. Even though not systematically assessed, there was also no apparent difference in the numbers of apoptotic glandular cells.

4.3.5.2. Endometrial stroma

In specimens from patients treated with either dose of asoprisnil, no decidual changes were identified except in one sample from the uterine fundus. In contrast, decidual changes were evident in 43% of sections from the placebo-treated group. Asoprisnil appeared to induce increased stromal compactness when compared to placebo. The stroma was assessed as compact in 0-14% of samples from the placebo group, 30-60% after 10mg asoprisnil and 45-70% after treatment with 25mg asoprisnil.

4.3.5.3. Vasculature

Two different vascular morphological features were observed with increased frequency in the endometrial stroma of asoprisnil-treated patients. These consisted of

leashes of thin-walled vessels and clusters of thick-walled muscularized vessels, similar to those seen in the stroma of endometrial polyps.

In the placebo group, aggregates of thin-walled vessels occurred in 14-29% of samples from uterine fundus and mid-corpus but not in the isthmus. At least one such aggregate was found in 30-50% of specimen from fundus or mid-corpus after treatment with asoprisnil and in 20-40% of samples from the isthmus. Clusters of thick-walled vessels were not identified in any samples from the placebo group. Assessing the specimen from uterine fundus and mid-corpus, this vascular morphological feature was found in 20-30% after administration of 10mg asoprisnil and in 45% with 25mg asoprisnil. In the uterine isthmus, these clusters were seen in 10 and 30% of samples following treatment with 10mg and 25mg asoprisnil (Table 4.6.). Although these thick-walled vessels are reminiscent of the appearances in endometrial polyps, there are distinct differences. In endometrial polyps, these clusters typically occur within a rounded, projecting polypoid mass with gland crowding and collagenous changes to the stroma. Administration of asoprisnil for 12 weeks does not lead to gland crowding and the stroma is not notably collagenized. On the contrary, the endometrium remains flat and often thinned.

Non-endometrial tissue was available for assessment of vascular beds in this study. 12 ovaries from 8 patients, 15 Fallopian tubes from 10 patients and 10 cervices were removed at the time of hysterectomy and were examined. No specific histological abnormalities were observed, and particularly no similar vascular changes as described above occurred in non-endometrial tissues.

4.4. Discussion

The profound clinical effects of asoprisnil on menstrual bleeding pattern are accompanied by distinctive changes to endometrial morphology. This phenomenon has previously been appreciated[137] and prompted the development of a list of diagnostic criteria for classification of morphological changes induced by PRMs. The criteria were defined by a group of expert gynaecological pathologists, as the endometrial features encountered did not comply with the categories within the existing compendium of pathological diagnoses. Previous studies into the effects of asoprisnil on endometrial morphology relied on biopsies obtained by Pipelle aspiration after treatment for 28 days[137]. For this study, full thickness endometrial samples with underlying myometrium were available following administration of asoprisnil for 12 weeks. The biopsies were mostly well oriented and undisrupted by the sampling procedure facilitating detailed morphological assessment.

The findings in this study were consistent with previous descriptions of PRM effects on endometrium. The majority of endometrial samples from asoprisnil-treated patients showed a non-physiologic secretory effect. This category is specific for the PRM effect and was assigned to only one placebo-treated sample. These particular morphological appearances were evident to a similar extent in all three uterine locations (fundus, mid-corpus and isthmus) but were more common after exposure to the higher dose of 25mg asoprisnil. They are not usually seen in normal cycling endometrium or any described condition and have been hypothesized to reflect the partial PR agonist/antagonist effect of asoprisnil[129].

Histological features in both endometrial glands and stroma define the non-physiologic secretory effect. The glandular architecture was similar to the tortuosity seen in the normal secretory phase, but secretory activity or cytoplasmic vacuolation was rare. Notably, the glands were often cystically dilated, but even though there was nuclear stratification, mitotic activity was very rare and even absent in the samples exposed to the higher dose of 25mg asoprisnil. Cystic glandular dilatation is commonly associated in the minds of histopathologists with simple endometrial hyperplasia. However, as part of the features of the non-physiologic secretory effect, the dilated glands not only showed a paucity of mitotic activity but also absence of glandular crowding. The cystic changes often involved a focus of one or two glands in a field of non-dilated glands. Appearances of glandular dilatation may also commonly be found in inactive endometrium in the peri- or postmenopause. In such cases, cystic dilatation is not considered indicative of secretory activity and may indeed be a consequence of a lack of secretory activity leading to occlusion of the gland opening to the surface.

Total endometrial thickness after treatment with asoprisnil for 12 weeks was not increased, and the trend (even though not statistically significant) was for the mean endometrial thickness to be less than in the placebo group. Overall, no histological changes were found to give cause for concern about the development of malignant or premalignant disease. Each sample was specifically assessed for features of glandular cytological atypia, but none were identified. In some cases, there was a superficial resemblance to simple cystic hyperplasia due to the occurrence of dilated glands. Notably, any other features of simple hyperplasia were lacking and the glandular architecture certainly did not suggest complex hyperplasia. Comparison of mitotic activity in this study is limited as most patients in the placebo group were in the secretory phase of their cycle at the time of hysterectomy, when mitoses are not expected to be present. However, the level of mitotic activity in the asoprisnil-treated groups was notably low, which is consistent with previous findings in non-human

primates describing an endometrial antiproliferative effect[123]. It is important that this is recognized amongst histopathologists so that endometrial samples from patients treated with asoprisnil are not misdiagnosed as hyperplastic on the basis of cystically dilated glands.

Cystic glandular dilatation has also been found in previous studies with mifepristone, when a significant proportion of samples were classified as hyperplastic[105,162]. These early findings were subsequently reviewed with the conclusion that many cases had been inappropriately assigned to the category of simple hyperplasia, as no other features consistent with this definition could be identified[108]. Cystically dilated glands with inactive epithelial appearances are a morphological feature commonly occurring with administration of both mifepristone and asoprisnil. Similarly, the endometrial antiproliferative effect has been consistently described with both compounds[124,194,195]. However, there are also significant differences in the morphology of endometrium after exposure to different PRMs. With mifepristone, the glands appear less tortuous and more tubular suggesting absent agonist activity on the PR with a pronounced antagonist effect. The glandular tortuosity seen in asoprisnil-treated endometrium likely reflects the partial PR agonist effect in addition to the antagonist action.

The most characteristic stromal effect in this study involved the endometrial vasculature. Additionally, there was increased compactness without any decidual changes. It has been hypothesized that the mechanism of action of asoprisnil may be related to the vascular morphological changes. Notably, no specific effect on endometrial vasculature has been identified after treatment with mifepristone. Administration of asoprisnil caused a more frequent occurrence of both aggregates of thin-walled vessels as well as clusters of vessels with thickened muscularized walls compared to controls. No similar changes were found in the limited range of non-endometrial tissues examined in this study. This specific effect on the endometrial vasculature may be mediated via the perivascular cells, which are known to express PR and have been described to be pivotal in the control of menstruation[9]. Perivascular cells are intimately apposed to the endothelial cells of the spiral arterioles and are thought to initiate vasoconstriction-vasodilatation cycles in response to the falling progesterone levels in the late secretory phase.

The clusters of thick-walled vessels are reminiscent of those found in the stroma of endometrial polyps. In asoprisnil-treated endometrium, the aggregates of thin-walled vessels are probably a precursor of these clusters of muscularized arterioles. It is possible that an effect of asoprisnil on the perivascular cells mediates an active process of angiogenesis. Alternatively, the development of these vascular aggregates

may be analogues to the stromal vessels in endometrial polyps. These are thought to represent focal areas of endometrium, which are not shed at the time of menstruation and therefore persist for several cycles to form polyps. Similarly, asoprisnil treatment suppresses menstrual bleeding, and hence endometrium is not shed, which may cause these morphologically abnormal vessels to develop. However, there are no other similarities between polyps and asoprisnil-treated endometrium, which remains flat and mostly thin with absent gland crowding and no prominent stromal collagen. Notably, polyps developed very infrequently following administration of asoprisnil, and in this study only one small polyp was found in one patient who had been taking asoprisnil 10mg.

4.4.1. Limitations of the study

Similar to the findings in the clinical study, the effect of asoprisnil on endometrial morphology appears profound and notable after administration for 12 weeks. This study does not allow any conclusions, whether these morphological changes will be maintained or further modified with prolonged exposure to asoprisnil. Of particular interest would be the assessment of glandular morphology, endometrial thickness and mitotic activity to provide assurance regarding the safety of long term administration of asoprisnil.

The applicability of the results in this study may be limited by the presence of benign gynaecological pathology in all patients. All patients in this study had at least one uterine fibroid and had initially presented with symptoms associated with fibroids such as heavy menstrual bleeding. Therefore, the endometrium cannot be considered functionally normal and the results should be interpreted with caution. Every effort was made to sample the endometrium with at least 10mm distance to the nearest fibroid. However, vascular changes are known to occur within the endometrium in the presence of uterine fibroids, even though in this study, all endometrial biopsies obtained at screening were reported as histologically normal.

The results in this part of the study are all descriptive and based on the findings of a single expert pathologist. As this is the first study assessing full thickness endometrial specimen following administration of asoprisnil, it may be regarded as a pilot study. Further studies including statistical inferences regarding the effects of asoprisnil on endometrial morphology are indicated, in particular for measurements of endometrial thickness. The numbers in this study were too small to compare each asoprisnil group individually to placebo, and it would be interesting to assess

whether the trend for asoprisnil to reduce endometrial thickness may be significant in a larger sample group.

The effect of asoprisnil on vasculature has only been described in endometrium and appears to be tissue-specific. None of the non-endometrial tissue specimen in this study demonstrated any similar changes or other histological abnormalities. However, these specimen were obtained incidentally and not specifically for the purpose of assessing asoprisnil-related morphological changes.

4.5. Conclusion

Unique changes in endometrial morphology have been demonstrated following administration of 10 or 25mg asoprisnil once daily for 12 weeks. For the first time, a detailed assessment of endometrium exposed to asoprisnil has been carried out on full-thickness samples obtained at hysterectomy. Most notable were the effects on glandular appearances as well as the vascular changes in the stroma, which appeared to be specific to the endometrium. It is important for histopathologists to be aware of these unique effects of PRMs when examining endometrial samples from patients treated with such compounds to avoid misclassification. In particular, some appearances are reminiscent of simple hyperplasia or polyps but other concomitant features are clearly inconsistent with these entities. The mechanism of action of asoprisnil and particularly its effect on the suppression of menstrual bleeding is still not clarified but may be mediated by the perivascular cells and may be related to the altered vascular morphology.

CHAPTER 5

ENDOMETRIAL PROLIFERATION

The data presented in this chapter have previously been peer-reviewed and published in the manuscript:

Wilkens J, Williams ARW, Chwalisz K, Han C, Cameron IT and Critchley HOD
Effect of asoprisnil on uterine proliferation markers and endometrial expression of the tumour suppressor gene, PTEN
Human Reproduction 2009; 24(5): 1036-44

5.1. Introduction

The endometrial antiproliferative effect of progesterone receptor modulators (PRMs) as described in the previous chapter was discovered soon after their first development and initially with mifepristone[191]. With the progesterone receptor (PR) antagonistic properties of PRMs, this was quite an unexpected finding, as it may have been hypothesized that unopposed oestrogenic effects potentially induce endometrial proliferation. Since then, the antiproliferative effect has been consistently described with mifepristone and other PRMs in humans and non-human primates[123,196]. Even with administration of pure PR antagonists such as ZK 137 316, the endometrium appeared atrophic[197]. This phenomenon has alternatively been termed functional non-competitive anti-oestrogenic effect, but the exact mechanism of action has still not been fully elucidated[123]. Until this is understood, endometrial safety remains a potential concern, particularly when long-term administration is considered, even though studies of endometrial morphology have been reassuring. Occasionally occurring features such as cystic glandular dilatation, which are partially reminiscent of hyperplasia, have actually been associated with low mitotic indices in both endometrial glands and stroma[135]. In particular, there have been no reports of premalignant or malignant morphological changes following administration of PRMs[136].

In addition to morphological studies, proliferation may be further assessed by evaluating the expression of proliferation markers such as Ki-67 and anti-phospho-histone H3 (PH3). The nuclear antigen Ki-67 is a marker, which may be detected in all phases of the replicating cell (G1, S, G2 and M) representing the growth fraction of a cell population[198,199]. More recently, it has been described to play a role in ribosomal RNA synthesis as well as mitosis and may therefore also be expressed in non-proliferating cells[200]. The Ki-67 index is less specific than the mitosis-specific marker PH3, which is only expressed during the actual phase of mitosis (M). When proliferation of normal endometrium in different phases of the menstrual cycle is

assessed using various proliferation markers, they all show a high proliferation index during the proliferative phase with a significant decrease in the secretory phase. There is a high correlation between the direct mitotic count and the PH3 count whilst the Ki-67 index shows the same trend but is less specific[14].

Further to the effect of PRMs on endometrial proliferation, it is of particular interest whether there is any potential to increase the risk of carcinogenesis. PTEN (phosphatase and tensin homologue) is a tumour suppressor gene product, which has been described as a gatekeeper for initiation of carcinogenesis in the endometrium[15]. Loss of PTEN function has been demonstrated to occur as an early event in endometrial carcinogenesis and has therefore been suggested as a biomarker for premalignant disease even in histologically normal endometrium[16,17]. Progesterone has previously been shown to play an important role in eliminating PTEN-deficient endometrial cells[18], and hence the effect of partial progesterone antagonists such as the PRMs on PTEN expression is important to evaluate.

A previous study with asoprisnil in cynomolgus monkeys described profound endometrial atrophy and also a decrease in the endometrial expression of the proliferation markers Ki-67 and anti-phospho-histone H3 (PH3)[195]. The objective of this study was to enhance the morphological studies described in the previous chapter and investigate in more detail the effects of asoprisnil on endometrial proliferation markers in humans. Ki-67 and PH3 immunohistochemistry was carried out in full thickness endometrial biopsies. Additionally, endometrial PTEN expression was evaluated to assess the carcinogenic potential of asoprisnil.

5.2. Materials & Methods

5.2.1. Immunohistochemistry

5μm paraffin sections were de-waxed, rehydrated, pressure-cooked for antigen retrieval and cooled as described in section 2.2.2.1. PTEN immunohistochemistry was carried out using the Bond-X Immunohistochemistry Staining System as outlined in section 2.2.2.2.

For Ki-67 and PH3 immunohistochemistry, tissue sections were washed in 0.01M phosphate buffered saline (PBS) before blocking endogenous peroxidase activity by immersion in 3% hydrogen peroxide for 10 minutes at room temperature. After a further wash in PBS, the appropriate protocols were followed for the two proliferation markers as described below.

After the final wash with PBST, the protocols for both proliferation markers Ki-67 and PH3 were followed by the addition of the chromagen 3, 3'-diaminobenzidine (DAB). The reaction was stopped with distilled water when nuclear staining was detected by inspection under the microscope. Harris's haematoxylin was used for counterstaining. The sections were then dehydrated and finally mounted with Pertex.

5.2.1.1. Ki-67

Slides were incubated in non-immune horse serum (NHS) in PBS for 20 minutes at room temperature in order to block non specific binding of the primary antibody. The primary antibody Ki-67 (1:100 dilution in NHS/PBS) was added and sections incubated for 30 minutes at 37°C. For the negative controls, the primary antibody was replaced with non-immune mouse IgG1 antibody at a matched antibody concentration (1:1000). Subsequently, slides were washed in PBS with added Tween 20 (PBST) before incubating in biotinylated horse anti-mouse antibody for 30 minutes at room temperature. Following another wash in PBST, an avidin-biotin-peroxidase complex (ABC-HRP) was applied for 30 minutes at room temperature before the final wash with PBST.

5.2.1.2. PH3

At room temperature, sections were incubated in avidin for 15 minutes, rinsed in PBS and then incubated in biotin for a further 15 minutes. To block non-specific binding of the primary antibody, slides were incubated in non-immune goat serum (NGS) in PBS with 5% bovine serum albumin (BSA) for 20 minutes at room temperature. The primary antibody anti-phospho-histone H3 (1:1000 dilution in NGS/PBS/BSA) was added and the slides incubated overnight at room temperature. For the negative controls, the primary antibody was replaced with non-immune rabbit antibody IgG at a matched antibody concentration (1:1000). Sections were washed in PBST before incubating in anti-rabbit envision kit for 30 minutes at room temperature and washing again with PBST.

5.2.2. Scoring

For assessment of PTEN immunostaining, a histoscore was applied as described in section 2.2.2.3. A separate histoscore was applied to surface epithelium, glandular epithelium, stroma, perivascular cells and endothelium, respectively.
Stereological methods were applied and varied for assessment of Ki-67 and PH3, respectively, as described below.

5.2.2.1. Ki-67

When the endometrial tissue sections were inspected after immunostaining for Ki-67, there was an impression of scanty staining in the majority of sections. It therefore appeared inappropriate to apply any histoscores, and in order to quantify the level of immunoreactivity, stereological methods were used as previously described[201].

The program used was Image-Pro plus 4.5.1 with Stereology-Pro 5.0 plug-in software in combination with an Olympus BH-2 microscope fitted with a Prior automatic stage. With the aid of the software, random fields were selected for counting and grids were placed over the fields at x200 magnification. All 432 intersections of a grid were defined as points, and all points falling over tissue were counted as one of the following categories: (a) – unstained epithelial cell, (b) – stained epithelial cell, (c) – unstained stromal cell, (d) – stained stromal cell, (e) – lumen (i.e. the empty space within glands or vessels). The proportion of tissue occupied by each of these categories was expressed as a percentage of total points counted (Figure 5.1.).

A percentage standard error (SE) value of less than 10% was aimed at to determine the number of fields to be counted. For all but four tissue sections, 10 fields (i.e. 4320 points) were sufficient to obtain a percentage SE of less than 10% for the categories of unstained cells and lumen. For three of the remaining tissue sections, 50 fields (i.e. 21600 points) and in one case 20 fields (i.e. 8640 points) were counted. The number of points occupied by stained epithelial or stromal cells was counted in a total of 50 fields for all tissue sections. According to the statistical formula for sample size calculations, 50 fields (or 21600 points) are sufficient to detect a proportion of 0.01% of stained cells assuming a null hypothesis of 0.00001%. Therefore, the point count was limited to 50 fields for all tissue sections[202].

5.2.2.2. PH3

The amount of PH3 immunostaining appeared very scanty indeed on first inspection of the slides. Therefore, the total number of stained epithelial and stromal cells in all sections was counted respectively, in order to quantify the positive immunoreactivity in endometrium. Stereological methods were used to determine and measure the area of endometrium in each section. The same program as for evaluation of Ki-67 immunostaining was used (Image-Pro plus 4.5.1 with Stereology-Pro 5.0 plug-in software) in combination with an Olympus BH-2 microscope fitted with a Prior automatic stage. The cell count was expressed as the number of stained cells per mm^2 endometrium.

Figure 5.1. Method of Ki-67 scoring

Stepwise illustration of the method of point count following immunostaining of the endometrium for Ki-67 antigen:

A Randomly selected field of microscopic view of immunostained endometrium at x200 magnification

B Application of a grid with 154 intersections

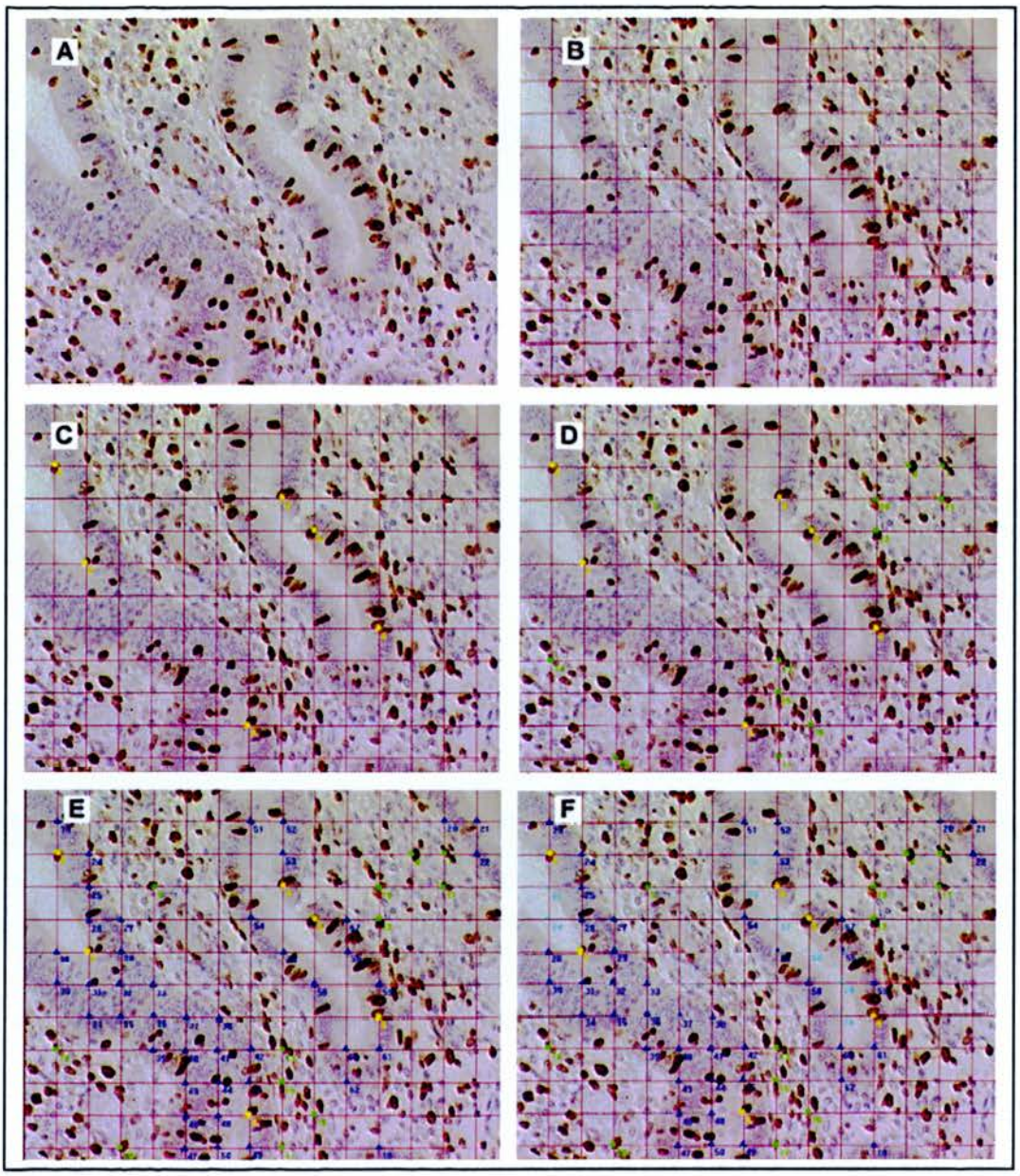
C Count of all stained epithelial cells overlying an intersection of the grid (6 yellow points = 3.9%)

D Count of all stained stromal cells overlying an intersection of the grid (12 green points = 7.8%)

E Count of all unstained epithelial cells overlying an intersection of the grid (44 dark blue points = 28.6%)

F Count of intersections of the grid overlying glandular lumen (8 light blue points = 5.2%)

All remaining points are counted as unstained stromal tissue (84 points = 54.5%).


A
B
C
D
E
F

5.2.3. Statistical analysis

Each group of asoprisnil treated subjects (10 and 25mg) was compared to a subgroup of placebo treated subjects who had undergone hysterectomy in the secretory phase of their menstrual cycle. The Wilcoxon's rank sum test was used for this analysis and significance was determined at 0.05 level using Hochberg's multiple comparison procedure.

5.3. Results

Endometrial expression of proliferation markers was low in all asoprisnil treated subjects as well as in placebo treated subjects in the secretory phase of the menstrual cycle.
There was high proliferation marker expression in the endometrium of the two placebo treated subjects who had undergone their hysterectomy in the proliferative phase.

5.3.1. Proliferation markers Ki-67 and PH3

The trend of the effect of asoprisnil on endometrial proliferation marker expression was suppressive. When Ki-67 immunostaining was quantified as described above, the median of grid points occupied by stained cells in endometrial epithelium and stroma was less than 2% in specimen from both asoprisnil- and placebo-treated subjects. Administration of asoprisnil led to a statistically significant and dose-dependent decrease in Ki-67 expression in endometrial stroma (Fig. 5.2.A) compared to the placebo treated subjects in the secretory phase of their menstrual cycle. The 95% confidence intervals indicated that according to this study, 10mg asoprisnil reduces the percentage of stromal cells expressing Ki-67 by at least –1.51 and 25mg asoprisnil by at least –0.83 (Table 5.1.). Ki-67 expression was not statistically significant different between treatment groups in glandular epithelium (Fig. 5.2.B).
Immunostaining for PH3 showed generally a very low count of stained endometrial cells in epithelium and stroma of both asoprisnil- and secretory phase placebo-treated subjects ($< 3/mm^2$). There was no statistically significant difference between treatment groups (Fig. 5.3. / Table 5.1.).

Proliferation marker expression	Treatment Group		
	Placebo / Secretory (N=8)	Asoprisnil 10 mg (N=12)	Asoprisnil 25 mg (N=11)
Ki-67 % Total points occupied by stained stromal cells			
Mean +/- SD	1.83 +/- 0.94	0.71 +/- 0.44	0.36 +/- 0.36
Minimum - Maximum	0.61 – 3.14	0.17 – 1.35	0.07 – 1.16
95% Confidence intervals Mean difference to placebo	N/A	-1.73 to -1.51	-2.11 to -0.83
P-value[a]	N/A	0.012[$]	<0.01[$]
PH3 Stained stromal cells per mm^2 endometrium			
Mean +/- SD	5.77 +/- 8.56	1.58 +/- 0.98	2.32 +/- 1.51
Minimum - Maximum	0.97 – 26.65	0.13 – 3.35	0.5 – 4.93
95% Confidence intervals Mean difference to placebo	N/A	-9.12 to +0.76	-8.91 to +2.01
P-value[a]	N/A	0.057	0.351

Table 5.1. Proliferation marker expression in endometrial stroma

Ki-67 expression is quantified by defining the percentage of total grid points occupied by stained stromal cells

PH3 expression is determined by counting all stained cells divided by the area of endometrium

[a] Each asoprisnil group is compared to placebo using Wilcoxon's rank sum test

[$] Denotes statistical significance at 0.05 level using Hochberg's multiple comparison procedure

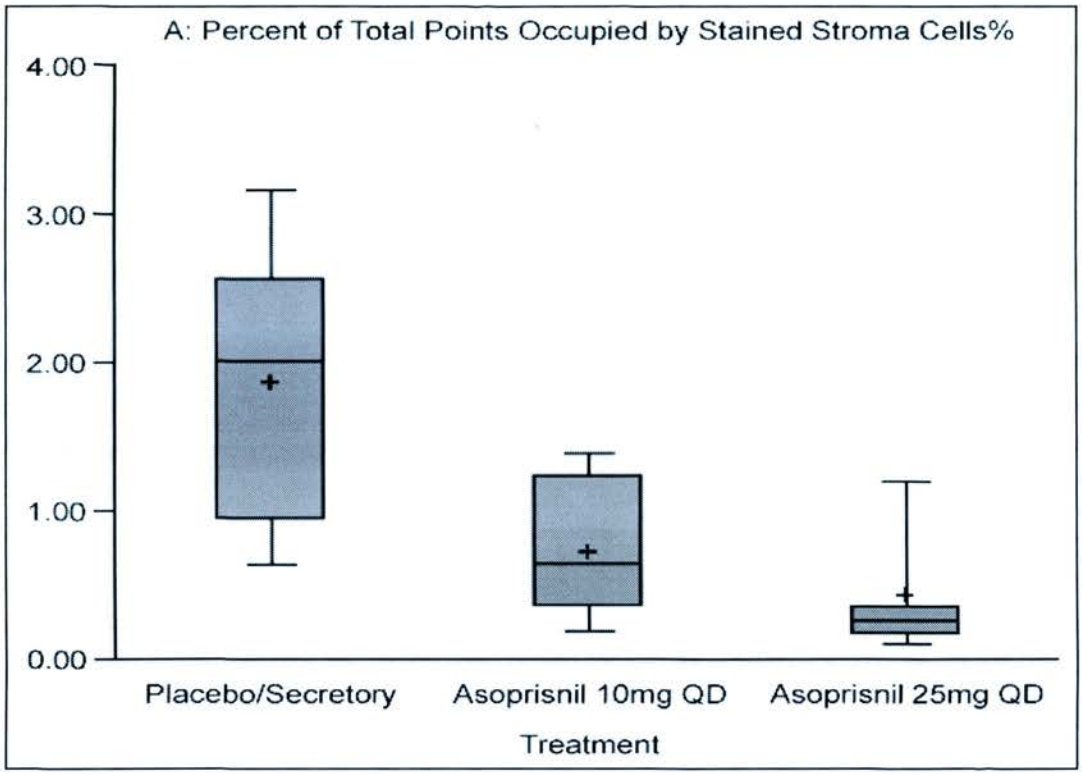
N/A = not applicable

Figure 5.2. **Ki-67 expression in endometrial stroma and glands following administration of asoprisnil**

A Ki-67 expression in endometrial stroma

B Ki-67 expression in endometrial glands

Ki-67 expression is quantified by percentage of grid points overlying stained glandular or stromal cells respectively, following the stereological projection of a grid over several views of a tissue section;
in the box plots, the box spans between lower and upper quartiles with the horizontal line within it representing the median; the whiskers extend to the minimum and maximum observations; the cross represents the mean; each asoprisnil group is compared to placebo (secretory phase) using Wilcoxon's rank sum test;
in stroma, both asoprisnil groups have a statistically significantly lower expression of Ki-67 than placebo using Hochberg's multiple comparison procedure at 0.05 level;
in glands, neither difference between an asoprisnil group and placebo is statistically significant


A: Percent of Total Points Occupied by Stained Stroma Cells%
4.00
3.00
2.00
1.00
0.00
Placebo/Secretory
Asoprisnil 10mg QD
Asoprisnil 25mg QD
Treatment

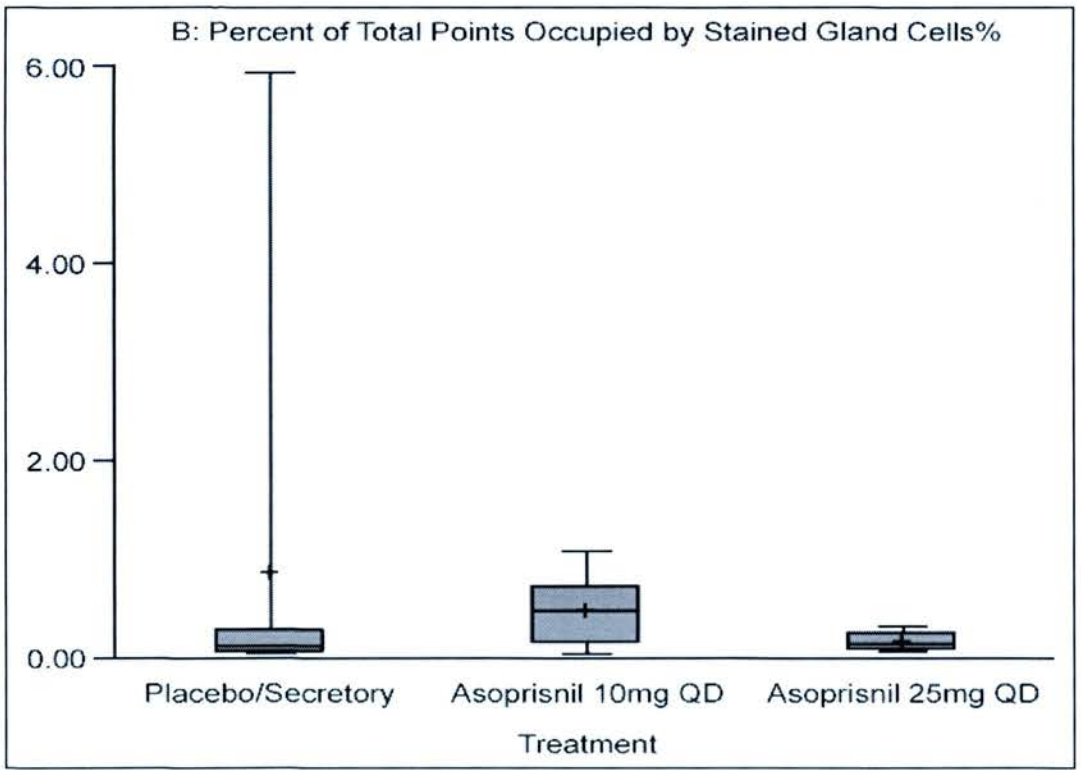
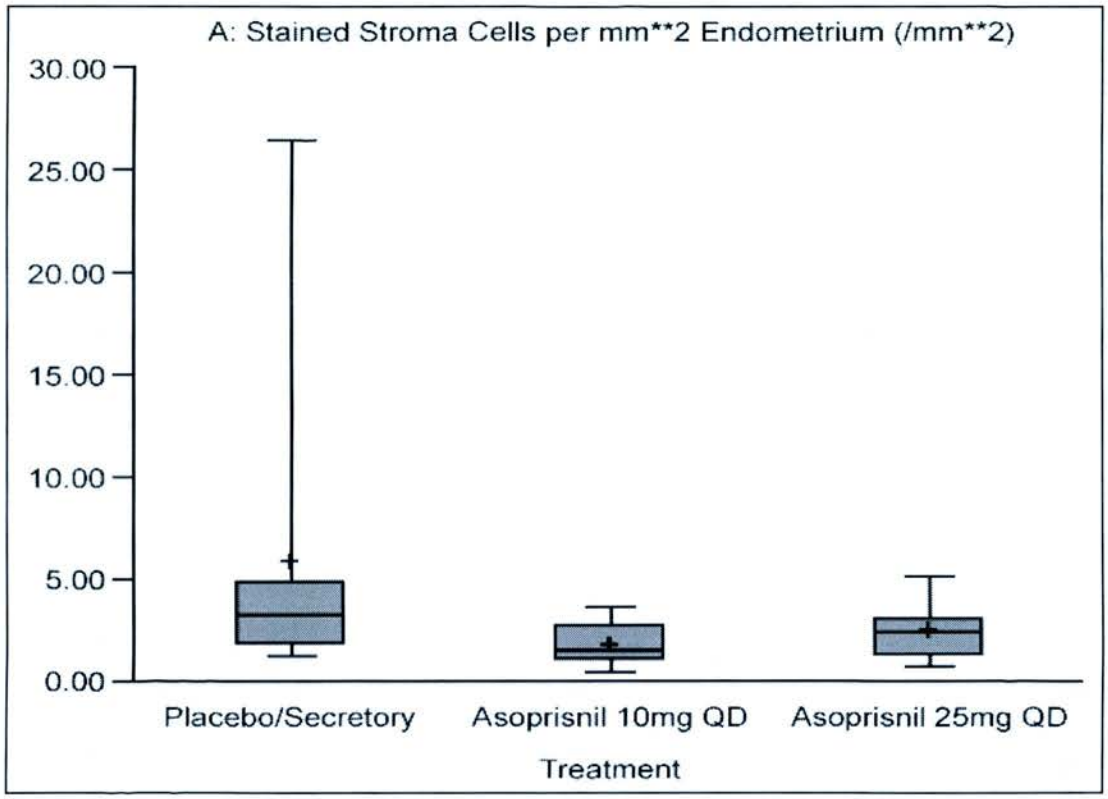

B: Percent of Total Points Occupied by Stained Gland Cells%
6.00
4.00
2.00
0.00
Placebo/Secretory
Asoprisnil 10mg QD
Asoprisnil 25mg QD
Treatment

Figure 5.3. PH3 expression in endometrial stroma and glands following administration of asoprisnil

A PH3 expression in endometrial stroma

B PH3 expression in endometrial glands

PH3 expression is quantified as stained glandular or stromal cells per mm^2 endometrium respectively following a count of all stained cells in the available tissue section and stereological measurement of the endometrial area;
each asoprisnil group is compared to placebo (secretory phase) using Wilcoxon's rank sum test;
none of the differences between asoprisnil and placebo groups is statistically significant


A: Stained Stroma Cells per mm**2 Endometrium (/mm**2)
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Placebo/Secretory
Asoprisnil 10mg QD
Asoprisnil 25mg QD
Treatment

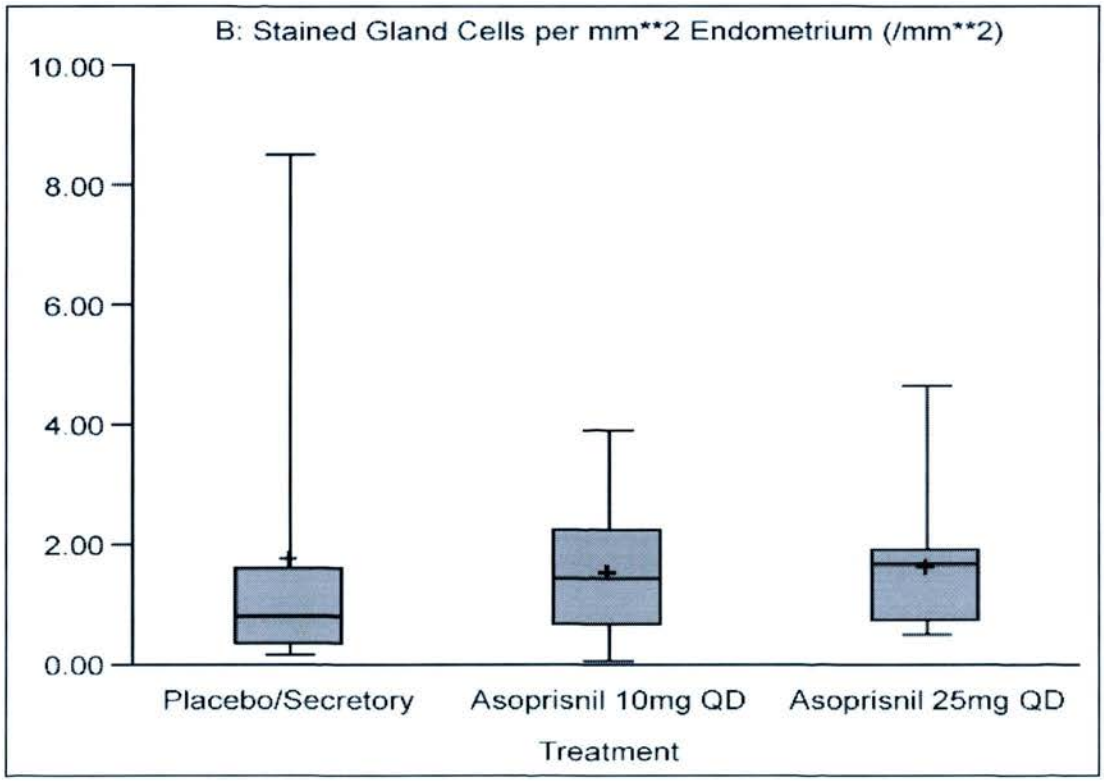
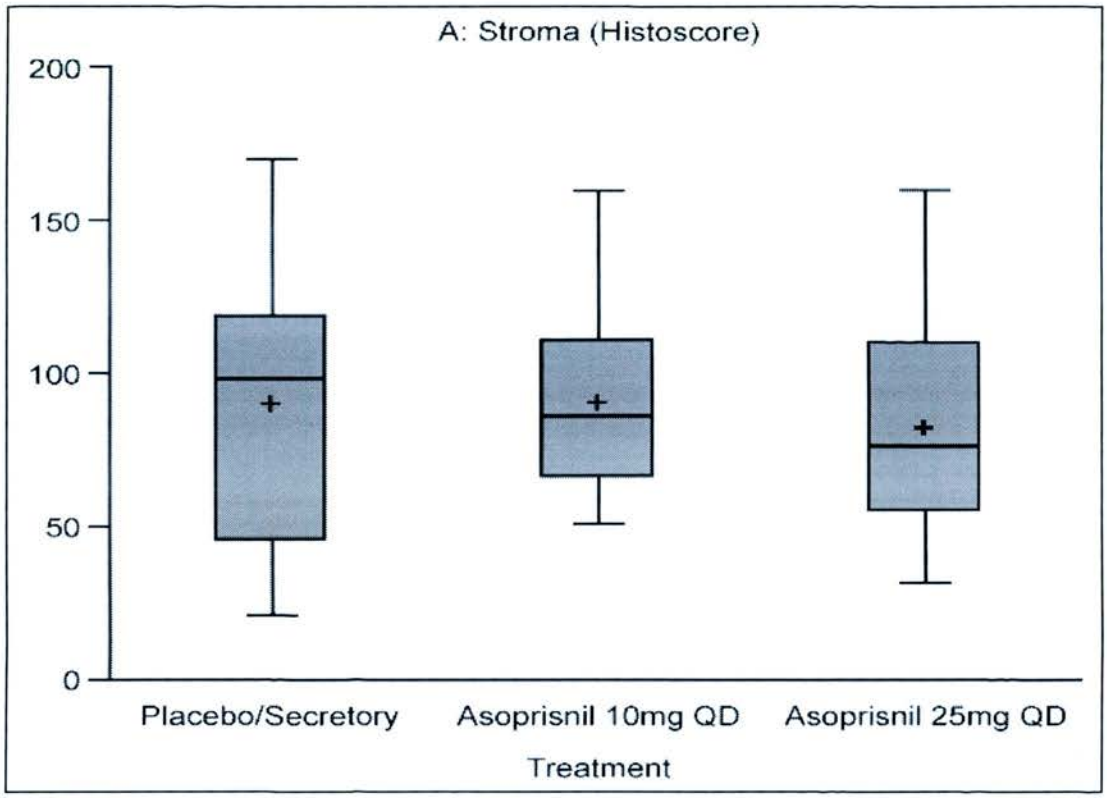

B: Stained Gland Cells per mm**2 Endometrium (/mm**2)
10.00
8.00
6.00
4.00
2.00
0.00
Placebo/Secretory
Asoprisnil 10mg QD
Asoprisnil 25mg QD
Treatment

Figure 5.4. PTEN expression in endometrial stroma and glands following administration of asoprisnil

A PTEN expression in endometrial stroma

B PTEN expression in endometrial glandular epithelium

PTEN expression is quantified by a histoscore (0 – 300); each asoprisnil group is compared to placebo (secretory phase) using Wilcoxon's rank sum test;
none of the differences between asoprisnil and placebo groups is statistically significant


A: Stroma (Histoscore)
200
150
100
50
0
Placebo/Secretory
Asoprisnil 10mg QD
Asoprisnil 25mg QD
Treatment

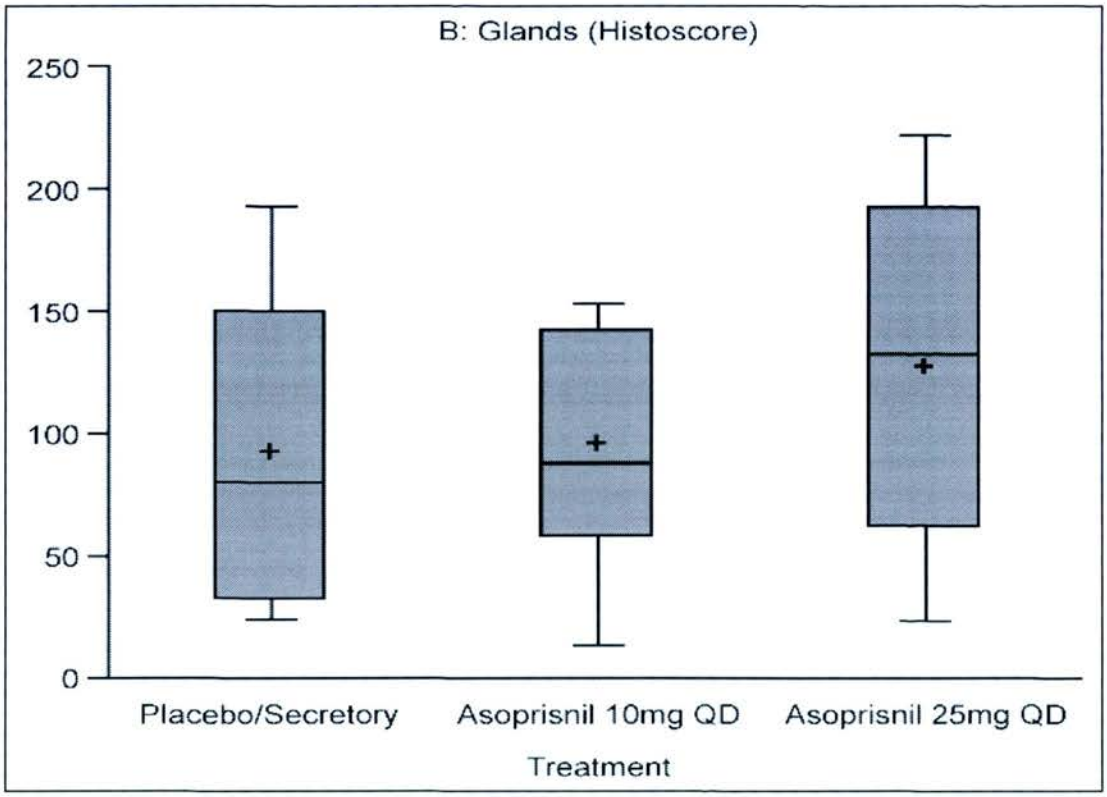

B: Glands (Histoscore)
250
200
150
100
50
0
Placebo/Secretory
Asoprisnil 10mg QD
Asoprisnil 25mg QD
Treatment

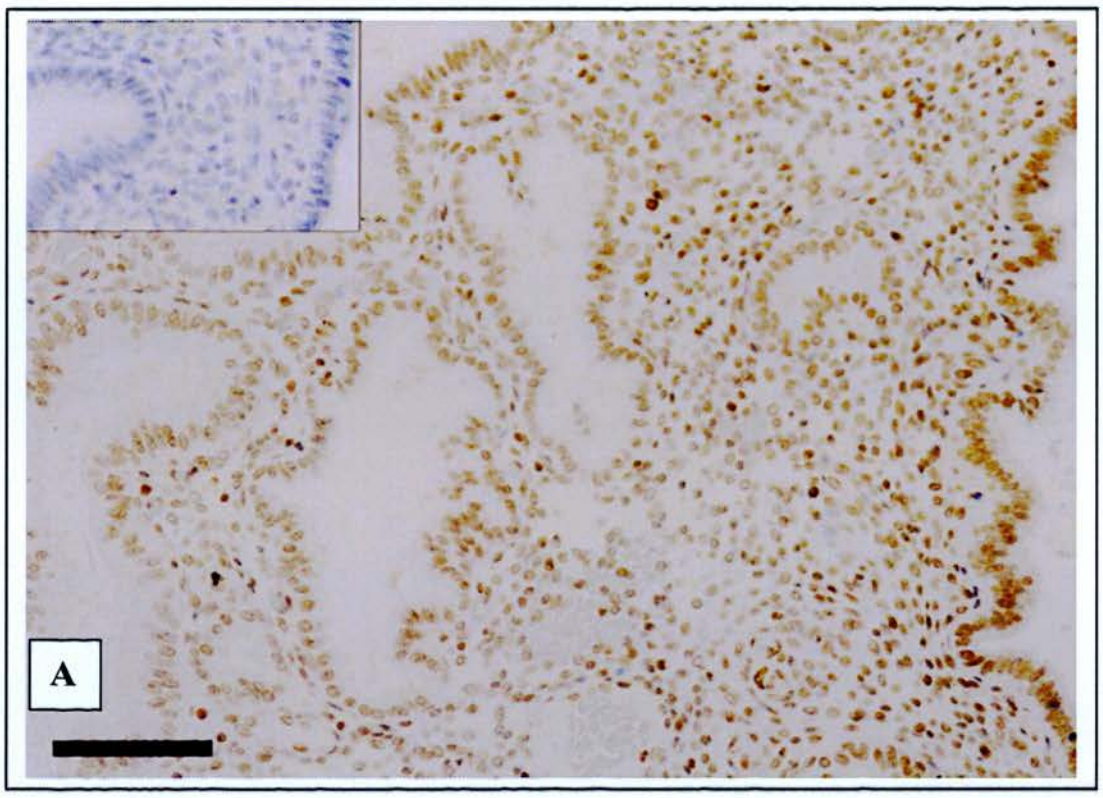
Figure 5.5. PTEN immunolocalisation in endometrium

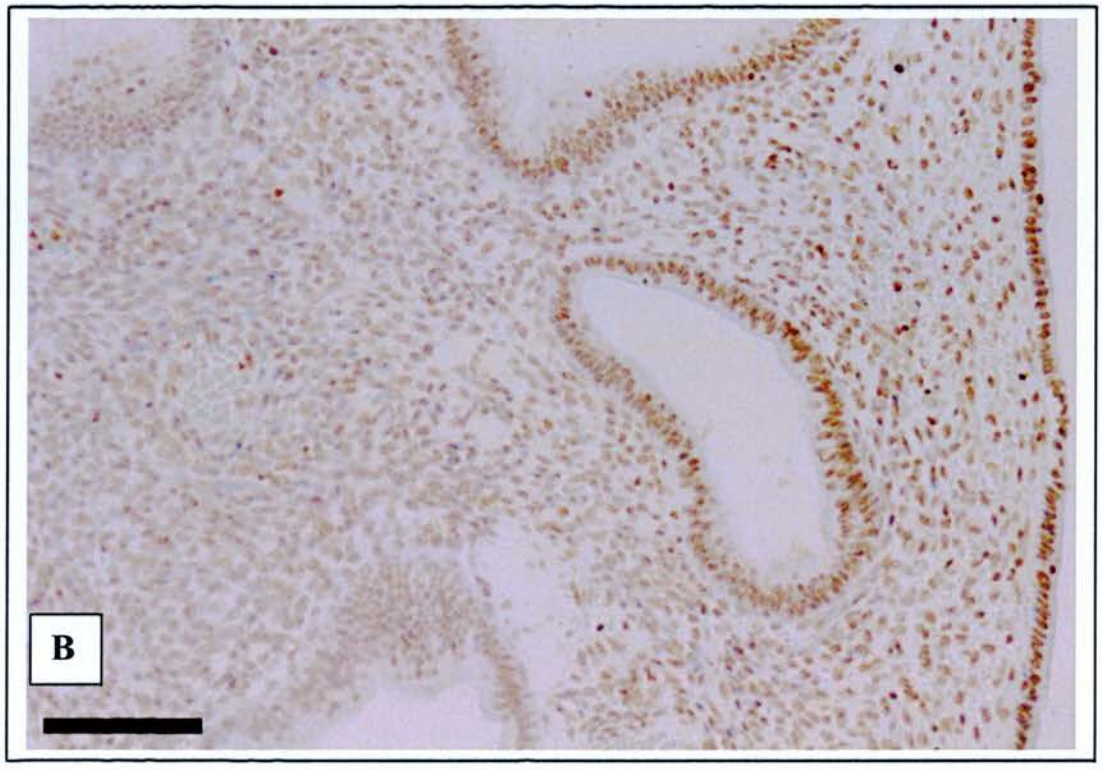
A PTEN immunostaining in endometrium from placebo group in secretory phase of menstrual cycle
Inset – Negative control

B PTEN immunostaining in endometrium from treatment group (asoprisnil 25mg)

Scale bar 100μm

(Previously published in:
Wilkens et al., Human Reproduction 2009; 1: 1-9)


A


B

5.3.2. PTEN

PTEN immunohistochemistry resulted in a nuclear staining pattern with similar intensity in glandular epithelium and stroma (Fig. 5.4.). The difference in PTEN expression between treatment groups was not statistically significantly different in any of the tissue compartments assessed. The staining intensity was strongest in the surface epithelium (median histoscores of 125-180) and only weak in endothelium and perivascular cells (median histoscores of 25-32.5 for perivascular cells and 30-45 for endothelium). In asoprisnil and placebo groups, there were inter-individual variations independent of the treatment received with some subjects showing very weak and others quite strong staining. However, there was no significant difference between treatment groups (Fig. 5.5.).

5.4. Discussion

The results of this study demonstrate that asoprisnil does not induce endometrial proliferation when administered daily for 12 weeks. A statistically significant difference in proliferation marker expression indicates an inhibitory effect in the endometrial stroma. This appears to be independent of ovarian oestrogen production, which is maintained at early proliferative phase levels in pre-menopausal women treated with asoprisnil[137,142].

Most patients in the placebo group of this study underwent hysterectomy in the secretory phase of their menstrual cycle. In these patients, endometrial proliferation marker expression was low in both glandular epithelium and stroma, whilst it was high in the two cases in which samples had been obtained in the proliferative phase. This is consistent with previous reports[14]. The low levels of proliferation marker expression in endometrium after 12 weeks of treatment with asoprisnil were comparable to samples from a secretory cycle phase. There was no statistically significant difference in PH3 expression between treatment groups, which is a likely reflection of the overall very scanty immunostaining. Expression of the less specific marker Ki-67 in endometrial epithelium was also not significantly different in the asoprisnil treated groups compared to the placebo treated group in the secretory phase. However, the finding of significantly decreased Ki-67 expression in the stroma of asoprisnil treated patients indicated a dose-dependent inhibitory effect on proliferation. There was no evidence of altered endometrial PTEN expression and specifically no suppression by administration of asoprisnil.

The effect of suppressed or unchanged proliferation marker expression after treatment with asoprisnil is consistent with the morphological findings described in the previous chapter and in previous trials. Endometrial hyperplasia or other appearances suggestive of an unopposed oestrogen effect have not been described with asoprisnil. Various doses of asoprisnil (5mg once daily to 50mg twice daily) have been administered to healthy premenopausal women for 28 days in one trial[137] and doses of 5, 10 or 25mg to women with uterine fibroids for 12 weeks in another study[142]. In both studies, Pipelle® endometrial biopsies were obtained following treatment with no findings of endometrial hyperplasia or cytological atypia. Distinct and unique morphological changes were noted reflecting the mixed progesterone agonistic/antagonistic properties of asoprisnil in the human endometrium but no overtly suspicious features. In this study, full thickness endometrial samples were obtained and examined with similar findings as previously described.

The effect of asoprisnil on proliferation marker expression has previously been studied in adult cynomolgus monkeys[195]. In a 39-week toxicity study in cynomolgus monkeys, the endometrium appeared morphologically atrophic with suppressed gland proliferation and stromal compaction. Some glands appeared dilated and cystic in the absence of any hyperplastic features[129]. However, contrary to the findings in the human, this study neither showed secretory changes in endometrial glands nor thickening of the wall of spiral arterioles. Studies in non-human primate models have been crucial in the discovery and development of SPRMs and have provided valuable insight into their possible mechanisms of action[132]. A distinct morphological response consistent with an antiproliferative effect has been seen in endometrium from monkeys as well as humans following exposure to SPRMs[191]. However, important differences between monkeys and humans have been highlighted indicating the need for caution when extrapolating results from non-human primate studies to the human. A different steroid receptor pharmacology of monkey and human endometrium may account for these discrepancies[132]. The balance between agonist and antagonist effect of asoprisnil may possibly be in favour of the agonist side in humans due to species differences in metabolic end products or due to species-specific cellular environments creating a different balance of co-activator and co-repressor expressions[203]. Specifically the findings of secretory endometrial gland changes and formation of thick-walled endometrial spiral arteries following treatment with asoprisnil in the human were unique and have not been observed in the monkeys. However, suppression of proliferation marker expression following asoprisnil exposure has been demonstrated in both human and non-human endometrium. Endometrial Ki-67 and PH3 expression was suppressed in cynomolgus

monkeys treated with 10, 30 or 90mg/kg asoprisnil for 90 days. The suppression was significant when compared to samples from the placebo group in the proliferative cycle phase[195]. Concordant with the findings in this study, there was no significant difference between asoprisnil treated monkeys and monkeys in the secretory cycle phase. In the monkey studies, there was no differentiation into separate tissue compartments (stroma and epithelium), whilst this study showed human Ki-67 expression to be significantly suppressed in endometrial stroma but not in the epithelium after 3 months asoprisnil treatment.

Ultimately, to assure safety of long-term administration of asoprisnil and other SPRMs, evidence is required that they do not induce endometrial carcinogenesis. The most common form of endometrial cancer is the endometrioid endometrial adenocarcinoma, which is specifically associated with the risk factor of exposure to unopposed oestrogen. In this type of cancer, the PTEN tumour suppressor gene is inactivated in up to 83%[15]. Both PTEN mutations and deletions are frequently found and have also been identified in precancerous endometrial tissue with endometrial intraepithelial neoplasia[16]. Decreased endometrial PTEN expression may indicate carcinogenic potential even in the absence of any histological changes. PTEN expression may be altered by exogenous hormones. This was demonstrated, when a group of peri- or postmenopausal women with endometrial hyperplasia, as seen in an endometrial biopsy, were treated with a progestin-impregnated intrauterine system (IUS), cyclic oral progestins or kept under surveillance only. Endometrial biopsies were repeated after three months, and the proportion of samples with absence of PTEN expression significantly declined in the group treated with the IUS. Hence, high doses of locally administered progestins appear to ablate latent endometrial precancers[18]. Systemic administration of progestins has also been shown to increase PTEN expression and induce the involution of precancerous endometrial lesions[204]. Consequently, a compound with partial progesterone antagonist activity may raise the concern of an unfavourable effect on PTEN expression and therefore on the potential to influence the predisposition to latent endometrial precancerous lesions. This was the reason to investigate the effect of asoprisnil on endometrial PTEN expression in this study, which found that PTEN expression remained unaltered after 12 weeks of treatment. This finding further supports the conclusion that the effect of asoprisnil on endometrium is antiproliferative and does not increase the risk of carcinogenesis. Morphologically, the endometrial glands may occasionally become cystically dilated in response to administration of asoprisnil. Low mitotic indices within these glands have already indicated that they are not associated with endometrial hyperplasia. This is further affirmed by the findings of low Ki-67 and

PH3 expression. In addition, maintained PTEN expression assures that the distinct morphological changes seen with SPRMs are not a feature to raise concerns about carcinogenic potential and are not pre-malignant.

It still has not been fully elucidated how the endometrial antiproliferative effect of SPRMs is mediated. The endometrial vasculature has been suggested as a principal target and particularly the spiral arteries[131]. The perivascular cells may have a pivotal role in mediating the effects on these vessels[132]. This hypothesis appears to be supported by the striking effect of asoprisnil on the formation of thick-walled spiral arterioles in humans. However, the absence of similar morphological vascular changes in non-human primate models suggests there may be another pathway. More recently the role of the endometrial androgen receptor (AR) has been emphasized as a potential mechanism of the endometrial antiproliferative effect[123,205].

5.4.1. Limitations of the study

Due to the overall very low prolilferation marker expression, all endometrial samples had to be assessed in some detail for quantification. Endometrial PH3 expression was particularly scanty, and the sample size in this study was possibly too small to demonstrate a significant effect as indicated by the wide confidence interval (Table 5.1.) Sample size was determined by the power calculation for the primary endpoint of the clinical study and therefore fixed for any further variables of investigation. The study into proliferation marker expression described in this chapter may be particularly limited by a lack of power as the expected differences would be very small. Even though the findings in this study appear reassuring regarding endometrial safety with administration of asoprisnil, no conclusions may be drawn regarding the effects of prolonged exposure beyond the duration of 12 weeks.

5.5. Conclusion

The results of this study have demonstrated that the endometrial antiproliferative effect of asoprisnil, which has previously been described morphologically, is accompanied by low proliferation marker expression. Expression of Ki-67 in endometrial stroma was significantly suppressed compared to placebo controls in the secretory menstrual cycle phase. Furthermore, this study showed that endometrial PTEN expression remains unchanged during asoprisnil treatment. Asoprisnil therefore did not appear to increase the carcinogenic potential within endometrial tissue.

CHAPTER 6

STEROID RECEPTOR EXPRESSION

6.1. Introduction

Sex steroids (oestrogen (E), progesterone (P), androgens and glucocorticoids) regulate reproductive functions, such as implantation and, in the absence of pregnancy, menstruation and endometrial repair. The effects of these steroid hormones are mediated via their respective nuclear receptors, and the role of various co-regulators has also been highlighted[25]. In human endometrium, it is well recognized that the spatio-temporal distribution of steroid receptors varies depending on the stage of the menstrual cycle[19].

Two forms of ligand-specific oestrogen receptors (ER) have been identified, ERα and ERβ. ERα appears to be the dominant isoform and is essential for normal uterine function[89]. ERα is also responsible for mediating the proliferative effect of E on endometrium[49]. Immunohistochemical studies in endometrium have demonstrated the expression of ERα within the nuclei of both glandular and stromal cells in the proliferative phase of the menstrual cycle. In the secretory phase, ERα declines in the functional layer of the endometrium but expression persists in the basal layer[206]. The second ER isoform, ERβ, has been discovered more recently[207]. Unlike ERα, ERβ is also expressed in the endometrial vascular endothelium, even though both isoforms are expressed in perivascular cells[29]. ERβ appears overall less abundant than ERα but is present throughout the menstrual cycle with a decline in only the glandular epithelium of the functional layer during the late secretory phase[29]. Whilst the exact role of ERβ still remains to be elucidated and may well involve some antagonistic action to ERα, its presence within the vasculature suggests it may mediate the effects of sex steroids on vascular remodelling[30].

The progesterone receptor (PR) has also been differentiated into subtypes. The two main isoforms, PR-A and PR-B, arise from a single gene. PR-A is the shorter subtype, which is devoid of 164 amino acids present at the N-terminus of PR-B. It is otherwise identical to PR-B but functionally distinct[19]. Studies in PR-A and PR-B knockout mice (PRAKO and PRBKO) have suggested PR-A to play a crucial role in decidualization. It is the dominant isoform in endometrial stroma and is required to oppose E-induced proliferation[89]. Immunohistochemical studies using antibodies, which recognize both subtypes, have demonstrated a peak in expression of PR in the late proliferative and early secretory cycle with a subsequent decline within the glandular epithelium, whilst stromal expression remains constant[56]. When a specific antibody to PR-B is used to differentiate the subtype expression, it appears that in the secretory phase, both subtypes are dramatically reduced in the glands and only PR-A is expressed in the stroma[64].

The endometrium is also a target tissue for androgens, which suppress E-dependent proliferation. In normal, cycling endometrium, the androgen receptor (AR) is only very weakly expressed in the glandular cells and is mainly localized in the stroma. It appears to be most abundant in the early proliferative phase with a subsequent decrease, particularly in the basal layer[24]. The expression of the glucocorticoid receptor (GR) in the endometrium has not been as widely studied, and the exact role remains to be determined. However, whilst it appears to be absent from glandular epithelium, strong GR expression has been demonstrated in the stroma and also in the endothelium. GR is present in those tissue compartments throughout the menstrual cycle and particularly in the proliferative phase[22].

The last three decades have seen the development of synthetic PR ligands, which do not have purely agonistic activity. First developed was mifepristone in 1981[99,101,104] followed by several other progesterone antagonists (PAs)[208]. More recently, a new class of PR ligands has been discovered exerting clinically relevant tissue-selective progesterone agonist, antagonist, partial or mixed agonist/antagonist effects on various target tissues depending on the biological action studied[139]. Within this class of selective progesterone receptor modulators (SPRMs), asoprisnil is the compound, which has undergone most pre-clinical and clinical evaluation. Its effects have been studied in women with symptomatic uterine fibroids[141] and endometriosis[139] as well as in healthy menstruating women[137]. The clinical effects of profound suppression of uterine bleeding in the presence of follicular phase E concentrations[141] and the distinct histomorphological changes of the endometrium with aggregations of unusually thick-walled spiral arterioles[135] have been described in previous chapters. Clinical evaluation was preceded by studies in Old World monkeys. The first evidence of the direct endometrial antiproliferative effects of asoprisnil was derived from studies in cynomolgus monkeys[131]. Further assessments during the developments of asoprisnil and other SPRMs have been performed in cynomolgus and rhesus macaques due to their similarities to humans with respect to hormonal regulation and morphological changes during the menstrual cycle[133]. Studies in these non-human primates have played a key role in the discovery and evaluation of SPRMs[132], even though important differences between non-human primate and human endometrial responses have also been observed, possibly due to variances in steroid receptor pharmacology and a different balance of co-activator and co-repressor expressions[203].

The effect of PAs on endometrial steroid receptor expression has been studied in rhesus macaques, where a striking up-regulation in ER, PR and AR in glands and stroma has been demonstrated[209]. Notable was the increase in ER, whilst

morphologically there was a clear inhibition of endometrial cell proliferation and growth. The glandular AR signal, usually only very weak in endometrial glands, was significantly enhanced by treatment with mifepristone, and stromal AR expression was also increased. This phenomenon has been confirmed in human endometrium with administration of the PA mifepristone[210]. The up-regulation of AR in endometrium exposed to mifepristone appears consistent in non-human primates and humans. A clinical study with daily low-dose mifepristone for 120 d showed a significant down-regulation of PR in both glandular epithelium and stroma after 60 and 120 d. ER expression was unchanged in surface epithelium and stroma and decreased in glands after 120 d, even though not significantly[194]. Low-dose mifepristone has also been shown to significantly up-regulate glandular GR expression in humans, when GR is usually localized specifically in endometrial stroma[211]. The aim of this study was to examine how asoprisnil modulates sex steroid receptor expression in human endometrium.

6.2. Materials & Methods

The methods of tissue collection, immunohistochemistry, quantitative reverse transcription-polymerase chain reaction (RT-PCR) and statistical analysis are all as described in chapter 2. Specifically examined were the endometrial expression of ERα, PR, AR, ERβ and GR. For the analysis of immunoexpression, a histoscore was applied as described in section 2.2.2.3[144]. In addition to the mRNA expression of the steroid receptors, the sex steroid co-regulators N-CoR, SRC-1, SRC-2 and SRC-3 were also studied by assays on demand (Table 6.1.).

Co-regulator	Accession Number
SRC-1	Hs00186661_m1
SRC-2	Hs00197990_m2
SRC-3	Hs00180722_m1
N-CoR	Hs00196920_m1

Table 6.1. Primer and probes of sex steroid receptor co-regulators for amplification by taq-man real-time Q-RT-PCR (Assay on demand)

6.3. Results

6.3.1. Immunoexpression of ERα, PR, AR, ERβ and GR

Endometrial ERα expression was significantly increased in both stroma and epithelium (Figures 6.1.A and 6.2.).

Endometrial PR expression was significantly down-regulated in the stroma following administration of 25mg asoprisnil with a lower 95% confidence limit of –27.7 compared to placebo/secretory. It was also suppressed in the perivascular cells by both doses of the drug compared to placebo. Conversely, PR expression was significantly up-regulated in both surface and glandular epithelium (CI 100-263 for 10mg asoprisnil and 100-269 for 25mg asoprisnil compared to placebo) (Figures 6.1.B and 6.3. / Table 6.2.).

There was significant up-regulation of AR expression in endometrial stroma, whilst the increase in the surface epithelium was only significant after 25mg asoprisnil. AR expression was not significantly altered in glandular epithelium (Figures 6.1.C and 6.4.).

Endometrial perivascular cells did not express ERα or AR, and endothelial cells did not express ERα, PR or AR. This pattern of sex steroid receptor expression did not change following exposure to asoprisnil. ERβ expression was significantly suppressed in the stroma by 10mg asoprisnil, whilst unaffected in other tissue compartments (Figures 6.1.D and 6.5.). The only effect of asoprisnil on GR expression was seen in endothelium with slight elevation after 10mg asoprisnil (Figure 6.6.).

6.3.2. Expression of nuclear steroid receptor and co-regulator mRNA

There was a statistically significant up-regulation of endometrial PR mRNA expression in both asoprisnil groups compared to placebo / secretory phase. The amount of PR mRNA normalized to the endogenous reference and relative to the placebo/secretory phase was 8.1 for the asoprisnil 10 mg group and 16.2 for the asoprisnil 25 mg group. There was also a trend for up-regulation of ERα and AR mRNA, although the differences between the placebo/secretory and asoprisnil groups only approached statistical significance. A statistically significant up-regulation in GR mRNA was seen only in the asoprisnil 25 mg group compared to placebo/secretory subjects. No statistically significant difference was observed for ERβ or any of the four nuclear co-regulators SRC-1, SRC-2, SRC-3 and N-CoR.

Progesterone receptor expression - Histoscore -	Treatment Group		
	Placebo / Secretory (N=8)	Asoprisnil 10 mg (N=12)	Asoprisnil 25 mg (N=11)
GLANDS			
Mean +/- SD	36.3 +/- 75.25	218.2 +/- 95.4	0.36 +/- 0.36
Minimum - Maximum	0 – 220	45 – 295	35 – 295
95% Confidence intervals Mean difference to placebo	N/A	+100.4 to +263.4	+100.2 to +269.0
P-value[a]	N/A	<0.001[$]	<0.001[$]
STROMA			
Mean +/- SD	104.4 +/- 55.45	62.1 +/- 49.56	31.8 +/- 38.55
Minimum - Maximum	25 – 190	5 – 160	5 – 140
95% Confidence intervals Mean difference to placebo	N/A	-89.4 to +5.1	-117.5 to -27.7
P-value[a]	N/A	0.085	0.002[$]

Table 6.2. Endometrial progesterone receptor expression

Progesterone receptor expression is quantified by applying a histoscore

[a] Each asoprisnil group is compared to placebo using Wilcoxon's rank sum test

[$] Denotes statistical significance at 0.05 level using Hochberg's multiple comparison procedure

N/A = not applicable

Figure 6.1. Endometrial expression of sex steroid receptors

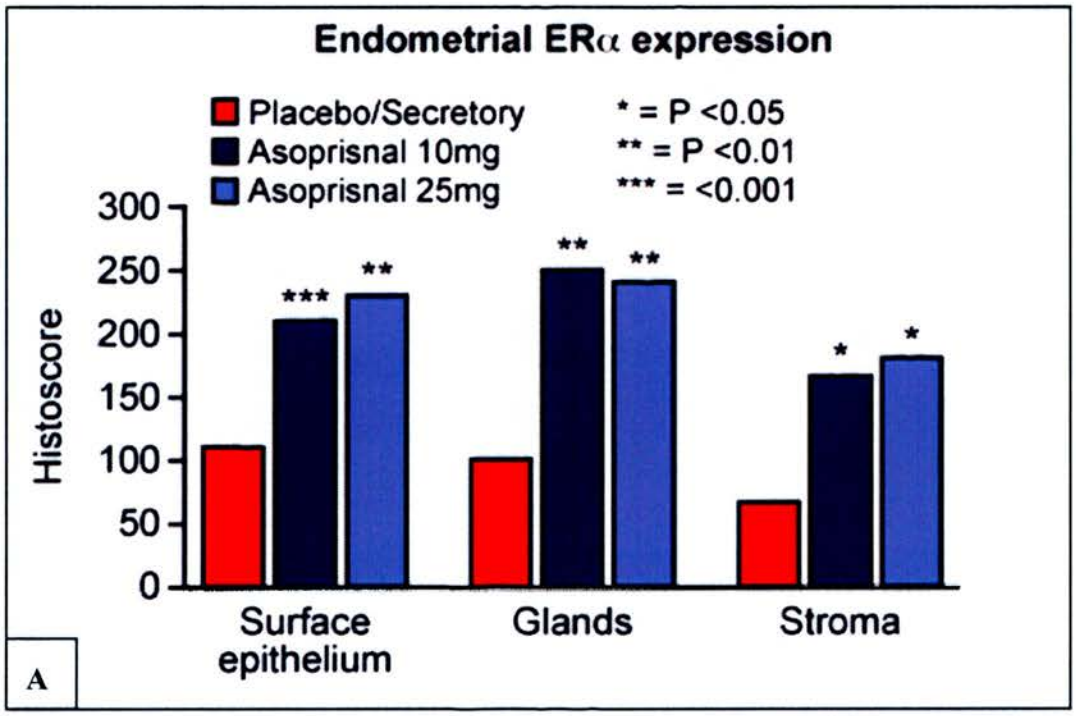
Endometrial expression of sex steroid receptors following administration of asoprisnil compared to placebo / secretory phase as assessed by applying a histoscore to surface epithelium, glands and stroma respectively;
the three treatment groups were compared by performing the Wilcoxon's rank sum test and significance determined at 0.05 level using Hochberg's multiple comparison procedure; significance is indicated by * = $P<0.05$, ** = $P<0.01$ and *** = $P<0.001$

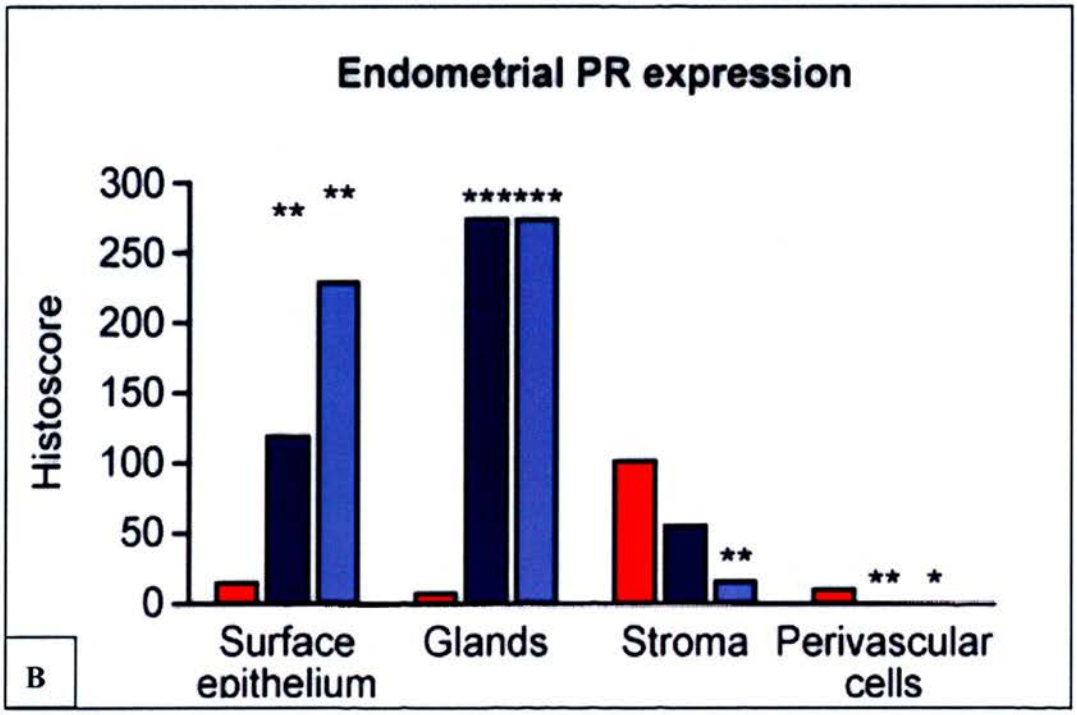
A Endometrial ERα expression

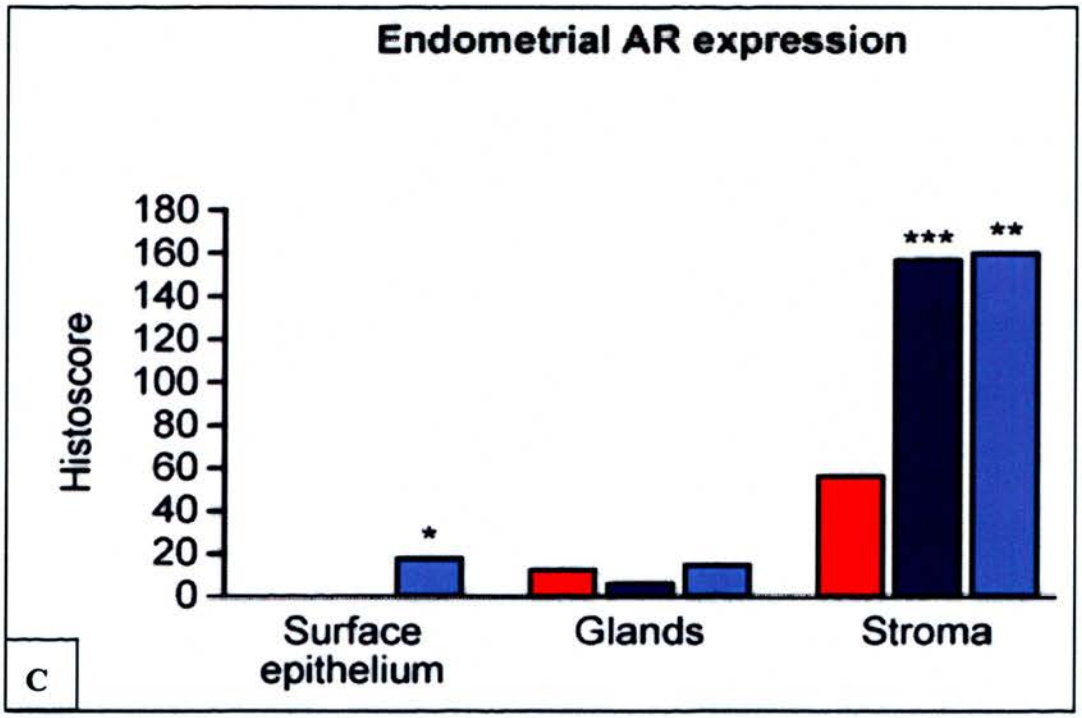
B Endometrial PR expression

C Endometrial AR expression

D Endometrial ERβ expression


Endometrial ERα expression
Placebo/Secretory
Asoprisnal 10mg
Asoprisnal 25mg
* = P <0.05
** = P <0.01
*** = <0.001
Histoscore
300
250
200
150
100
50
0
***
**
**
**
*
*
Surface epithelium
Glands
Stroma
A


Endometrial PR expression
Histoscore
300
250
200
150
100
50
0
**
**
******
**
**
*
Surface epithelium
Glands
Stroma
Perivascular cells
B


Endometrial AR expression
Histoscore
180
160
140
120
100
80
60
40
20
0
*
***
**
Surface epithelium
Glands
Stroma
C

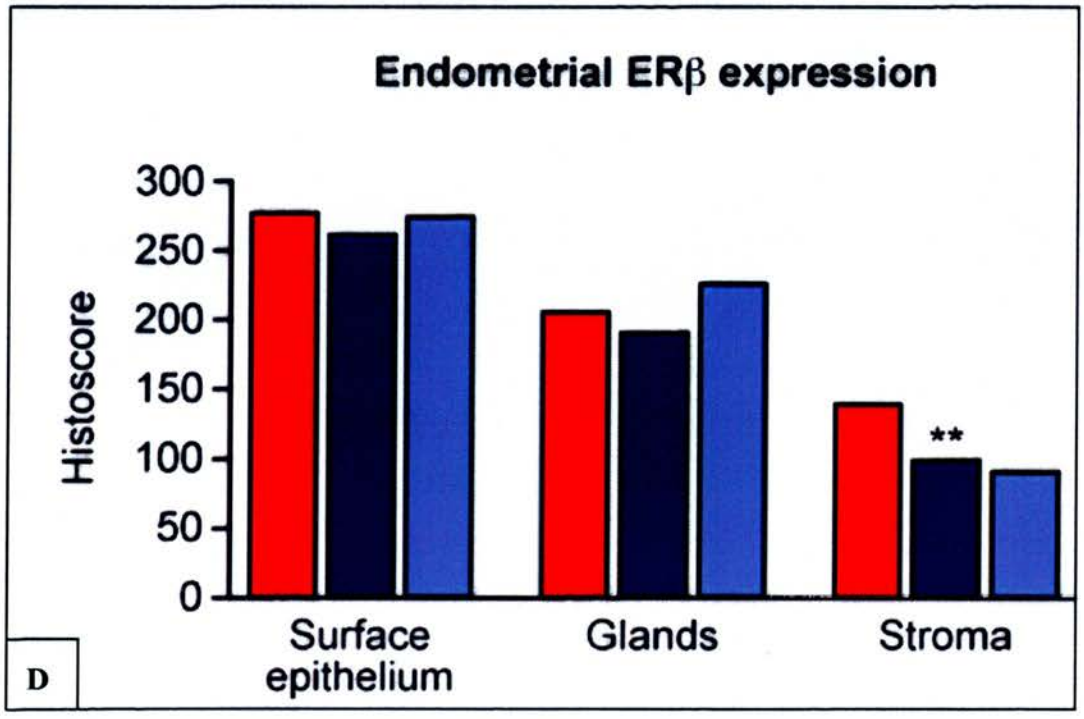
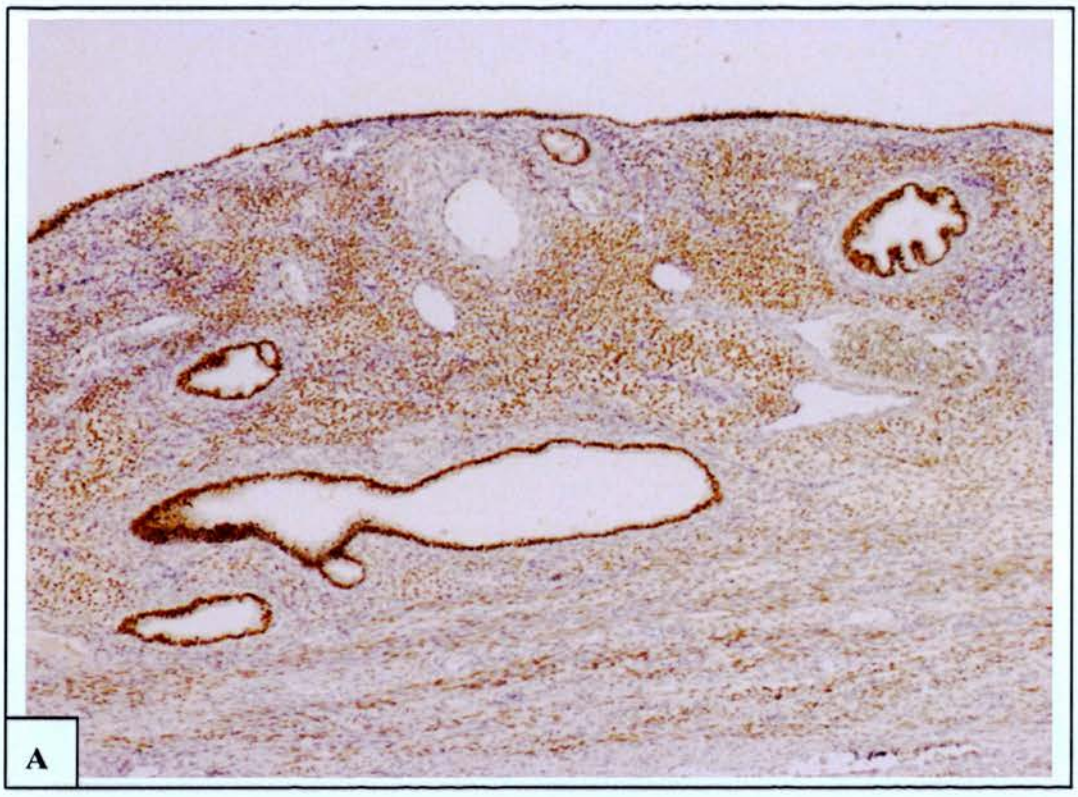

Endometrial ERβ expression
Histoscore
300
250
200
150
100
50
0
**
Surface epithelium
Glands
Stroma
D

Figure 6.2. Immunohistochemical expression of ERα

A Strong immunohistochemical expression of ERα in endometrial surface epithelium, glands and stroma following administration of asoprisnil 25mg

B Endometrial immunohistochemical expression of ERα following administration of placebo (secretory phase)


A

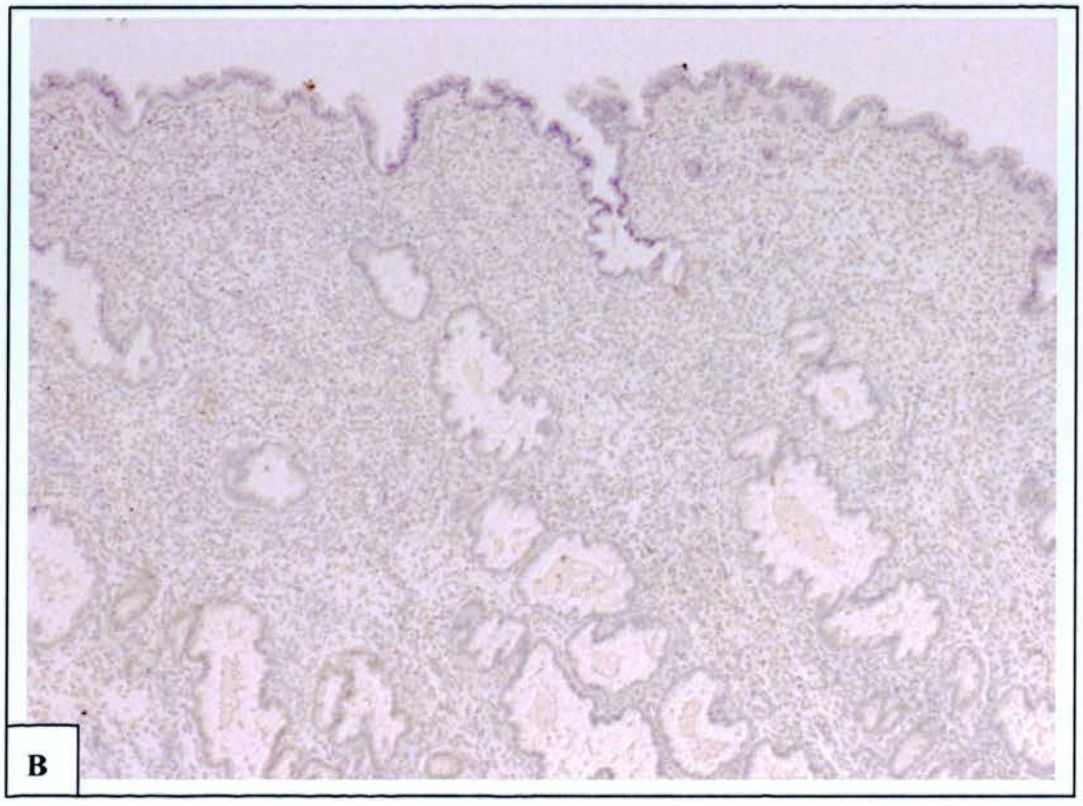
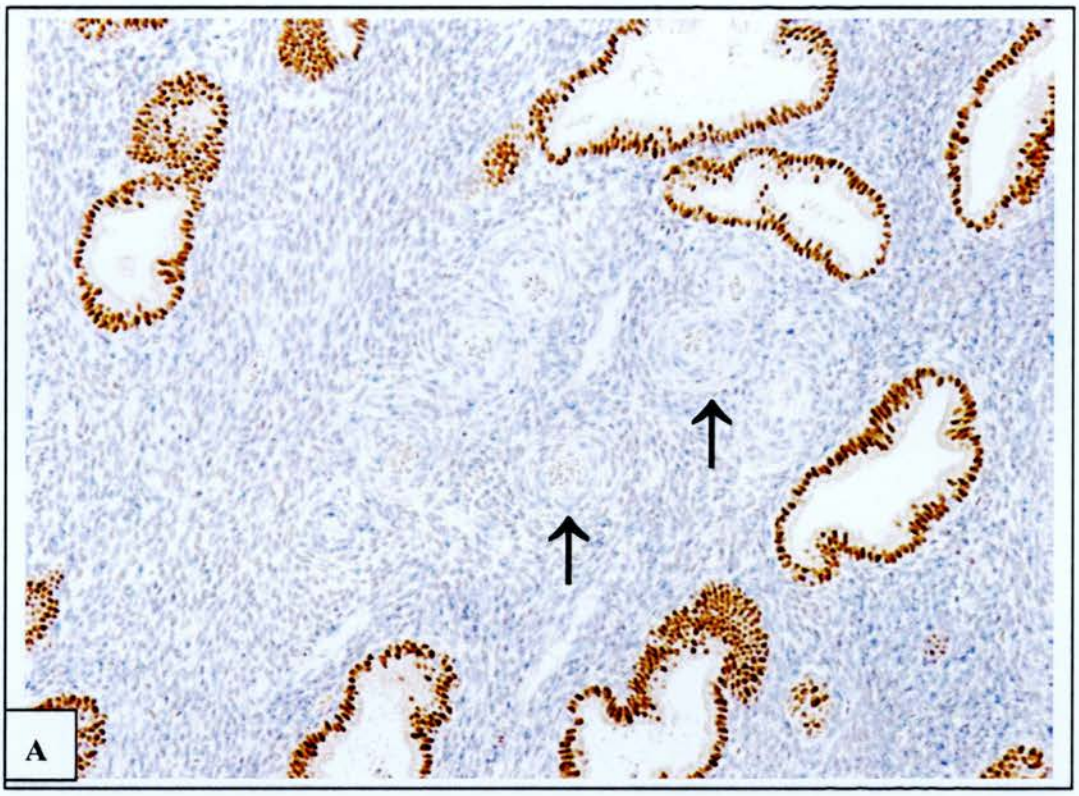

B

Figure 6.3. Immunohistochemical expression of PR

A Strong immunohistochemical expression of PR in endometrial glandular epithelium but only scarce expression in the stroma and notably in the perivascular cells (arrows) following administration of asoprisnil 25mg

B Endometrial immunohistochemical expression of PR predominantly in the stroma and present in perivascular cells (arrows) following administration of placebo (secretory phase)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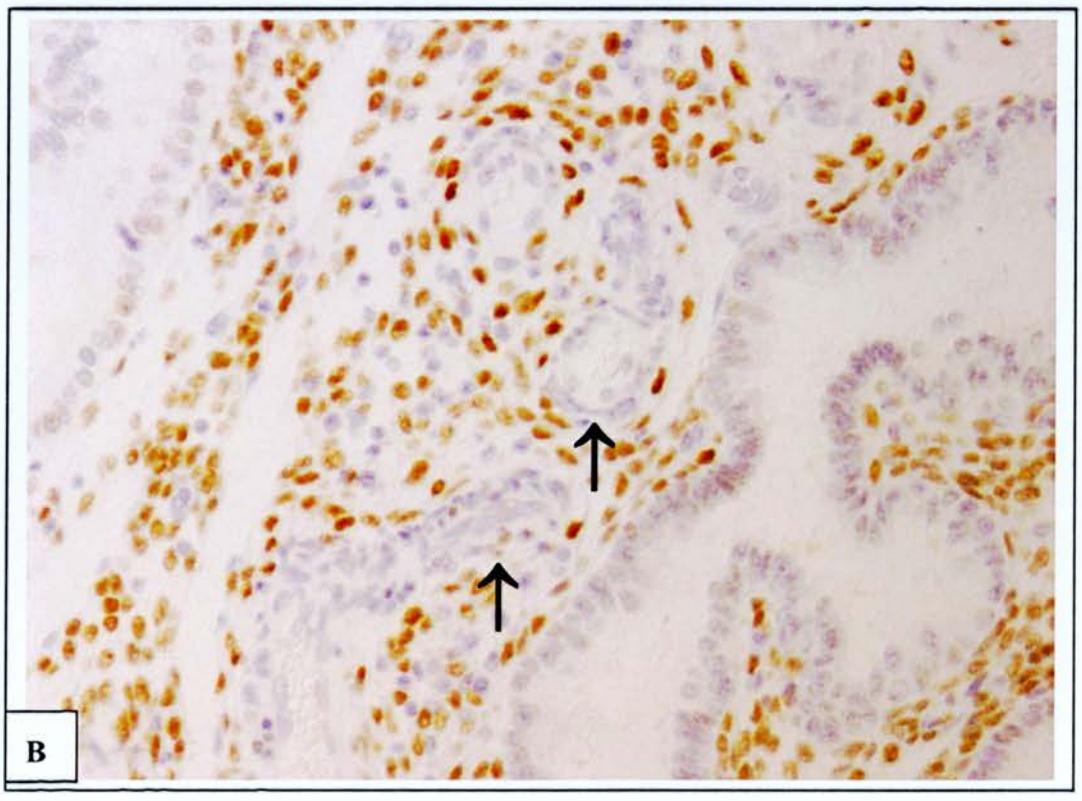
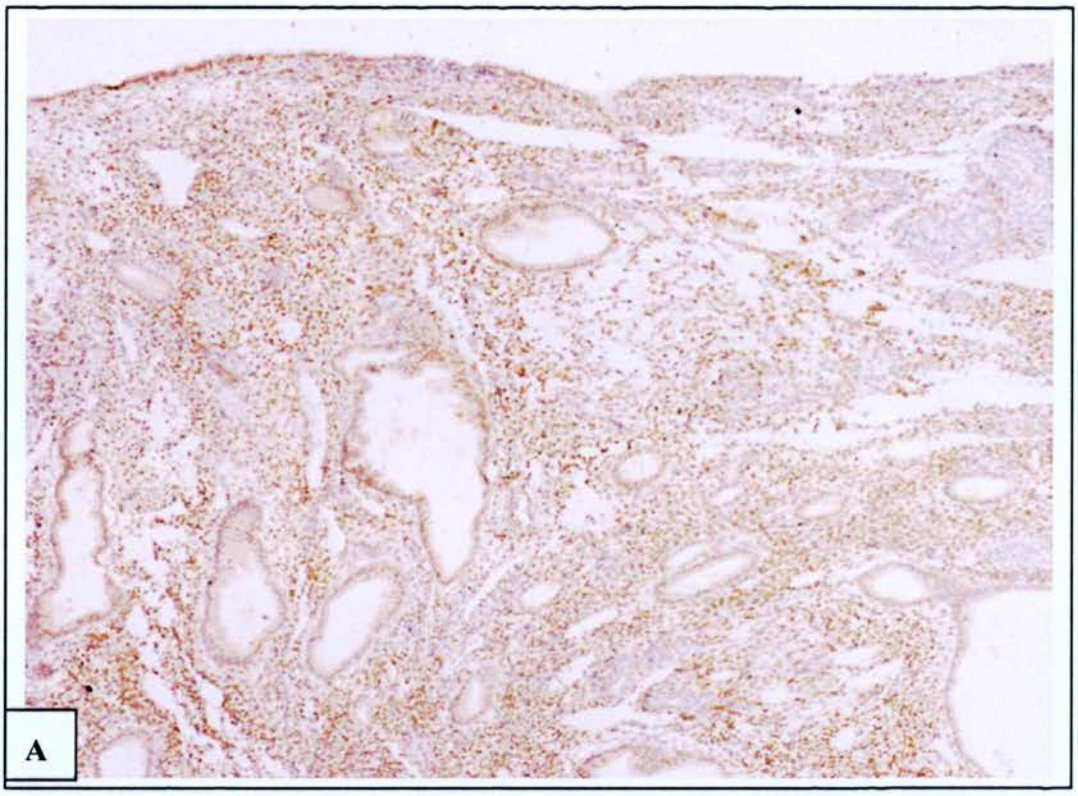

B

Figure 6.4. Immunohistochemical expression of AR

A Strong immunohistochemical expression of AR in endometrial stroma following administration of asoprisnil 25mg

B Endometrial immunohistochemical expression of AR following administration of placebo (secretory phase)


A

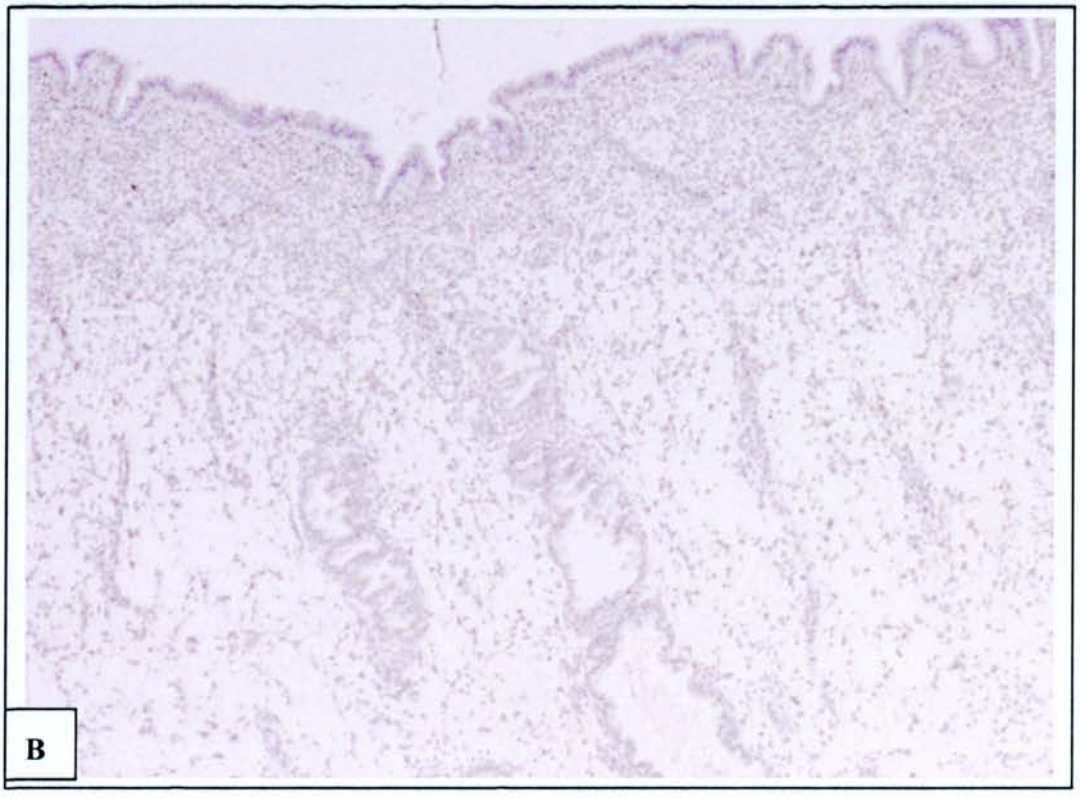

B

Figure 6.5. Immunohistochemical expression of ERβ

A Endometrial immunohistochemical expression of ERβ following administration of asoprisnil 25mg

B Endometrial immunohistochemical expression of ERβ following administration of placebo (secretory phase)


A

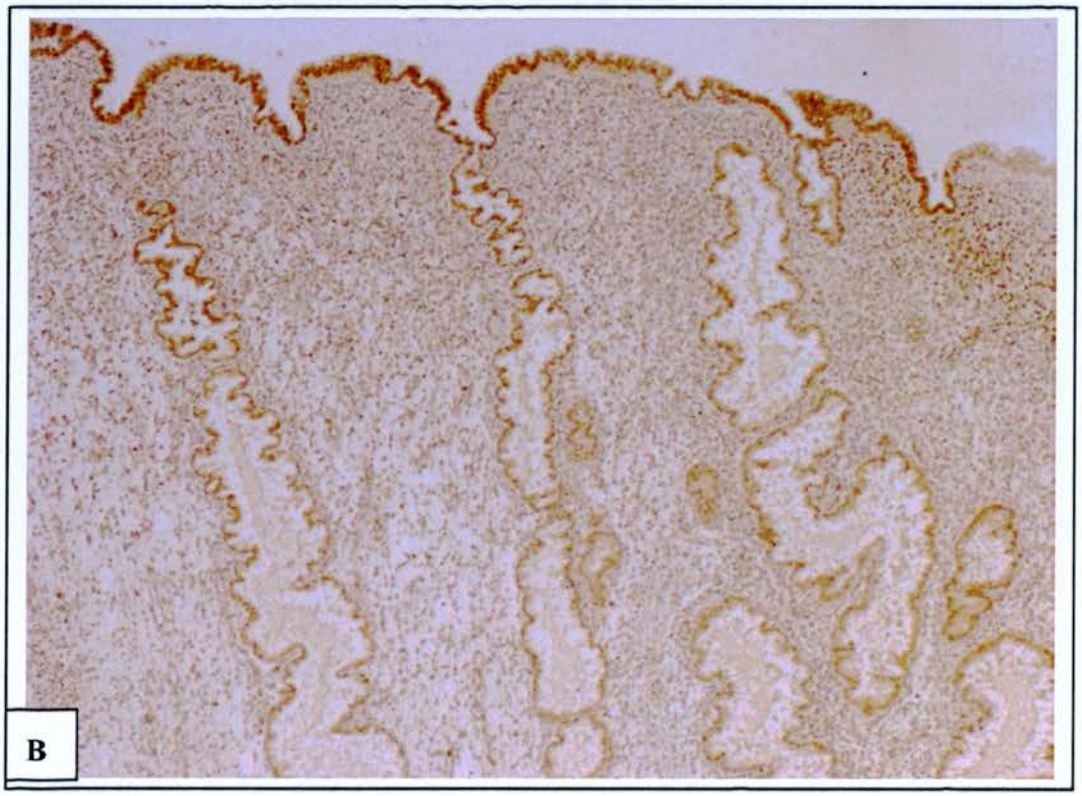
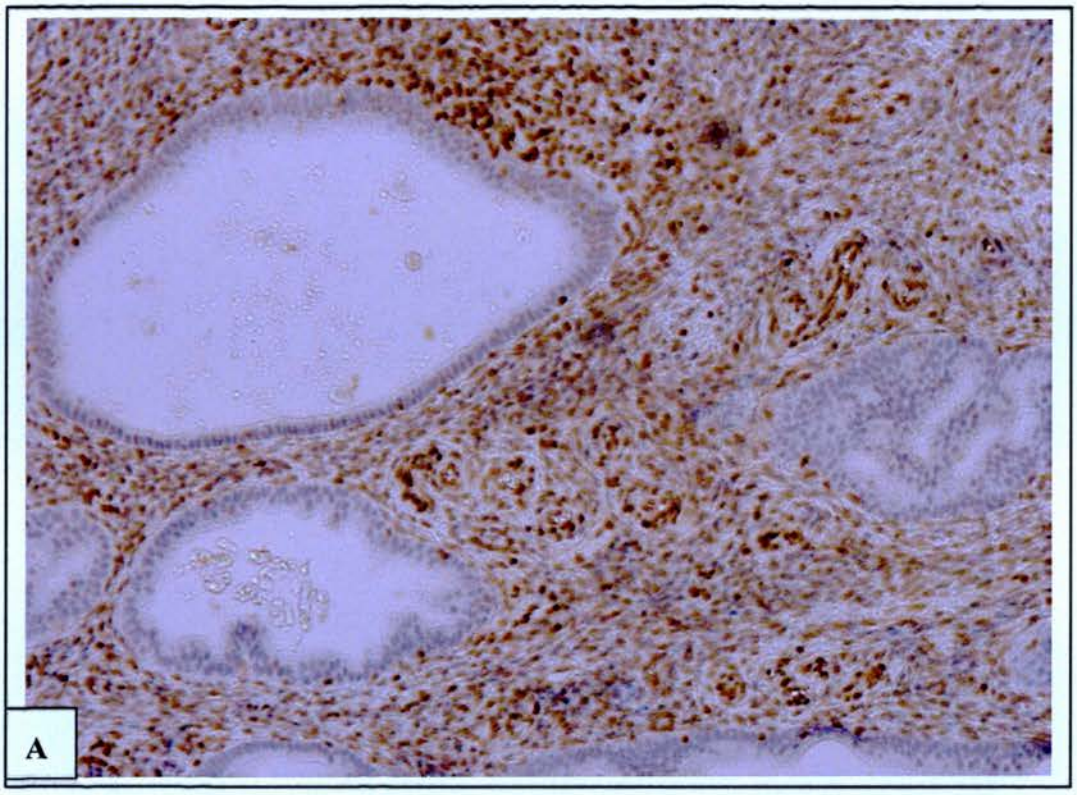
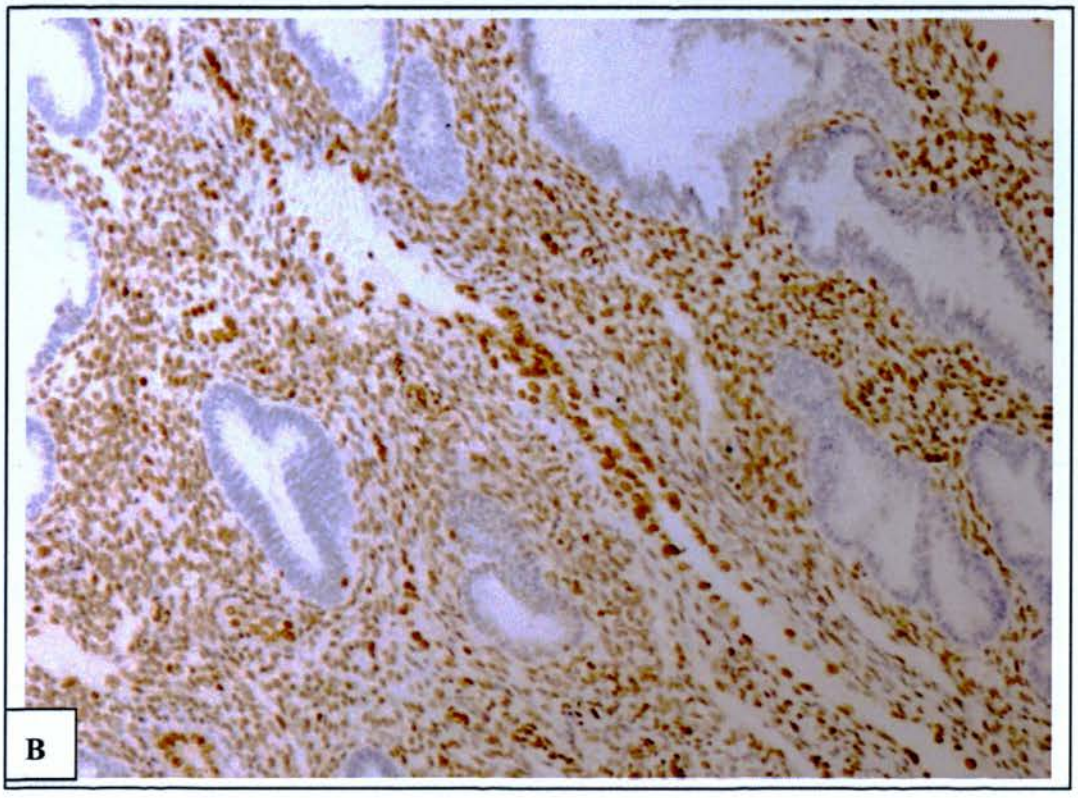

B

Figure 6.6. Immunohistochemical expression of GR

A Endometrial immunohistochemical expression of GR following administration of asoprisnil 25mg

B Endometrial immunohistochemical expression of GR following administration of placebo (secretory phase)


A


B

6.4. Discussion

Administration of asoprisnil significantly modulates human endometrial sex steroid receptor expression in both epithelium and stroma. In this study, there was clearly a differential effect on endometrial epithelium and stroma following administration of asoprisnil for 12 weeks. Whilst ERα expression was up-regulated in both epithelial and stromal compartments, PR expression was increased in the glands but suppressed in the stroma. Notably, there was also a down-regulation of PR in the perivascular cells. AR expression was considerably elevated in the stroma with a slight increase in surface epithelium, but no significant effect was observed in the epithelial glands.

The effect of asoprisnil on endometrial sex steroid receptor expression has previously been studied in cycling cynomolgus monkeys[195]. They were administered either saline or vehicle as controls or one of three doses of asoprisnil (10, 30 or 90mg/kg orally) daily for 90 days. Endometrial immunoexpression of ERα, PR and AR was assessed in full thickness samples. Whilst no significant effect on glandular epithelium could be demonstrated, there was significant down-regulation of PR and up-regulation of AR in the stroma. Stromal ERα expression was suppressed.

As in the studies with PAs[194,209], there are notable differences between the responses of human and non-human primate endometrium to administration of asoprisnil. Both species show a differential effect on epithelium and stroma. Stromal down-regulation of PR and up-regulation of AR is consistently found in both species. However, whilst ERα stromal expression is suppressed in the cynomolgus monkey, it is increased in the human. Epithelial expression of any sex steroid receptors was not significantly altered in the non-human primate, but both glandular ERα and PR were up-regulated in this study.

Non-human primate models have provided valuable insight into the possible mechanism of action of SPRMs[132], but variances in the responses between the non-human primate and the human endometrium have previously been highlighted. It is possible that the balance between agonist and antagonist properties of asoprisnil, influenced by the species-specific expression of co-regulators, is in favour of an agonist effect in humans[203]. This may account for the discrepancies between the human and cynomolgus monkeys.

The response in sex steroid receptor expression to the SPRM asoprisnil is different from the response to PAs in both human and non-human primate endometrium. A study in rhesus macaques showed a striking up-regulation in ER, PR and AR in glands and stroma by PAs[209]. In contrast, both ER and PR were down-regulated in the endometrial stroma by asoprisnil in the monkey. In humans, daily low-dose

mifepristone (5mg) administered continuously for 120 d has been demonstrated to down-regulate PR expression in both glandular epithelium and stroma, whilst ERα remains essentially unaltered except for a slight decrease in glandular epithelial expression[194]. Interestingly, the effect of a single higher dose of mifepristone (200 – 400mg) given in the luteal phase has been shown to have a different effect with up-regulation of both ER and PR in stroma as well as epithelium, similar to the findings in the rhesus macaque[212]. These observations highlight the importance of the duration of the exposure and the dose of administered compounds when evaluating effects on sex steroid receptor expression. The acute effect may differ from the observed changes following administration over a longer period. The endometrial response to asoprisnil administered for 12 weeks, however, did not resemble the response to either acute or chronic exposure to mifepristone. As shown in this study, asoprisnil suppressed stromal PR expression but significantly increased PR in the glands. As opposed to the equivocal effect of low-dose mifepristone on ERα, asoprisnil has been demonstrated to significantly up-regulate its expression in both stroma and epithelium.

The effect of mifepristone on sex steroid receptor expression appears to be similar in endometrial stroma and epithelium in both non-human primates and humans. This study has demonstrated that the endometrial response to asoprisnil is differentially modulated in various tissue compartments. This is particularly notable regarding the effect on PR expression in human endometrium, where both down-regulation in the stroma by 25mg asoprisnil and up-regulation in the epithelium are statistically significant compared to placebo following administration of the drug for 12 weeks. This differential effect on steroid receptor expression highlights the novel effect of asoprisnil on endometrium. Similar to the changes in endometrial morphology following administration of asoprisnil, which had not previously been described[135], the effect on sex steroid receptor and particularly PR expression appears to be unique to this SPRM. The exact significance of these modulations of sex steroid receptor expressions and the mechanism of action remain to be elucidated.

Consistently in non-human primates and in humans, both PAs and asoprisnil have been shown to up-regulate endometrial AR expression. This up-regulation is particularly striking in the stroma but notably also seen in the epithelium, where AR is usually only very weakly expressed. As the initially unexpected suppressive effect of PAs and SPRMs on endometrial proliferation resembles the atrophic effect induced by androgens, a role for the AR in the mechanism of action of PAs and SPRMs has previously been suggested[205]. The finding that treatment with the anti-androgen flutamide counteracts the antiproliferative effect of PAs in the primate

endometrium has confounded this hypothesis[123,213]. There is evidence that E-dependent growth factors essential for glandular mitosis and arterial growth are produced in the stroma[214], which may be suppressed by the increased AR expression. PAs have been proposed to block glandular cell mitosis by modulating E-dependent stromal-epithelial interactions via up-regulation of AR. A similar pathway may mediate suppression of vascular growth causing arterial degeneration and consequently atrophy and reduced endometrial blood flow. Whilst in normal endometrium, the effect of androgens on glands or vascular structures would be mediated indirectly through stromal AR, it is also possible that administration of PAs or SPRMs allows a direct effect through the up-regulation of epithelial AR expression[205].

There is some evidence that the primary targets, mediating the effects of PAs and SPRMs on primate endometrium, are the spiral arteries[131]. Indeed, morphological studies have found striking changes in the endometrial vasculature following administration of asoprisnil. Prominent aggregations of unusually thick-walled muscular spiral arterioles have been described, which do not feature in untreated endometrium[135]. In this context, it is notable that asoprisnil appears to suppress PR expression not only in the endometrial stroma but specifically in the perivascular cells. Previous studies in PR null mice have demonstrated evidence for a direct role of the PR in the regulation of the response to vascular injury and vascular smooth muscle cell proliferation. In vivo studies in the mice have suggested that the PR exerts an antiproliferative effect in vascular smooth muscle cells[215]. Modulation of PR expression in the perivascular cells by asoprisnil may mediate the effect on the vasculature and consequently its antiproliferative effect on endometrium. The findings of down-regulated PR expression in perivascular cells in this study and the previously described thickened spiral arterioles[135] appear consistent with the findings of the studies in the mice[215] and may provide insight into the role of PR in endometrial vascular remodelling and the mechanism of action of asoprisnil.

6.4.1. Limitations of the study

Despite the relatively small sample size, the confidence intervals for the mean differences of PR expression in glands after administration of asoprisnil compared to placebo, indicate a good reliability. Nevertheless, it would be appropriate to further confound these results with a study in a larger group of patients receiving asoprisnil for an extended period. Sex steroid receptor expression appears to be influenced significantly by the type, the dose and the duration of the compound administered,

and previous studies with mifepristone have yielded conflicting results depending on the study design. Prior to drawing firm conclusions, the reproducibility of the findings in this study would therefore need to be demonstrated. It is also possible that the response of the endometrial sex steroiod receptors to asoprisnil is modified by the presence of uterine fibroids in this cohort of patients.

6.5. Conclusion

We have demonstrated that asoprisnil has a significant effect on sex steroid receptor expression in human endometrium. Notably, asoprisnil exerts a differential effect in endometrial stroma and epithelium. This is most significant for the expression of PR, which is up-regulated in the glands by asoprisnil and down-regulated in the stroma. Interestingly, we have also shown a suppression of PR in the perivascular cells, indicating potential significance of PR modulation for the effects of asoprisnil on vascular remodelling.

CHAPTER 7

MODULATION
OF
LOCAL ENDOMETRIAL IMMUNE CELL FUNCTION

The data on microarray analysis described within this chapter have previously been summarized in the Final Report for TAP Pharmaceutical Products Inc entitled "A pharmacogenomic study on endometrium from subjects with uterine leiomyomata scheduled for hysterectomy following three month treatment with asoprisnil or placebo control" prepared by Mr Thorsten Forster, Mr Petter Storm, Mr Gary Rubin, Mr Tom Freeman and Professor Peter Ghazal from the Division of Pathway Medicine at the University of Edinburgh in October 2007. Figures 7.1. and 7.2. as well as Table 7.2. have been derived from this report.

The author was personally involved in the extraction of high quality RNA from endometrial samples collected at the time of hysterectomy from women in the clinical trial. With technical assistance from Ms Pamela Cornes, RNA was extracted and quality checks carried out until a sample of sufficient quantity and quality was available from each patient. This material was then passed onto the team in the Division of Pathway Medicine for microarray analysis.

7.1. Introduction

The most striking clinical effect of PRMs has been a profound suppression of uterine bleeding. The associated antiproliferative effect on the endometrium has been quite unexpected in a class of compounds with P antagonistic properties, and the mechanism of action still remains to be elucidated. A possible role for the AR has been described[205,213], but it has also been hypothesized that clinical and morphological effects of PRMs are mediated via the endometrial vasculature[103,131]. As outlined in chapter 4, asoprisnil leads to a characteristic morphological pattern in the endometrium, which has been termed "non-physiologic secretory effect". This unique pattern is defined in part by distinct vascular changes consisting of increased aggregates of thin-walled vessels but even more notably clusters of unusually thick-walled muscularized spiral arterioles. These prominent aggregations of thickened arterioles appear to occur exclusively in the endometrium. PR, the receptor asoprisnil ligates to, is normally abundantly expressed in endometrial stroma and notably in the perivascular cells[29,30]. If asoprisnil exerts its effects primarily via the endometrial vasculature, these characteristic vascular clusters may be a morphological indicator of this mechanism of action.

Over the last decade, microarray technology has been developed[216] and used to study endometrial function on a genomic level. A wide range of microarrays are commercially available and may be divided into the two categories of cDNA arrays and high-density oligonucleotide microarrays[217] with the latter providing a higher

degree of hybridisation specificity. This technology has enabled the analysis of expression of thousands of different genes at the same time, even though results should be validated using a different technique such as Q-RT-PCR or *in situ* hybridization. Microarray analysis has been applied to evaluate the normal function and cyclicity of endometrium, in particular with regards to endometrial receptivity during the window of implantation[218]. By studying the genetic events underlying the function of the normal endometrium, it has been envisaged that further insight might be gained into the processes leading to malfunction, and hence strategies for managing pathological conditions may be developed[219]. With this technology, endometrial tissue has been analysed in the different phases of the natural menstrual cycle[220] but also in response to hormones[221] and in pathological conditions such as endometriosis, uterine fibroids[222] and endometrial cancer. Specific efforts have been made to define the determinants of the receptive endometrium in order to facilitate infertility treatments. It has been in this context that the genetic response to the PA mifepristone, known to render the endometrium unreceptive, has previously been assessed[223,224].

The objective of this present study was to evaluate the effects of asoprisnil compared to placebo in patients with uterine fibroids in order to provide further insight into the potential mechanism of action. In particular, the hypothesis of asoprisnil exerting its effects via the endometrial vasculature was to be further explored. For the purpose of elucidating the mechanism of action, microarray technology using an Affymetrix high-density oligonucleotide microarray was applied to study the endometrial gene expression profiles of asoprisnil-treated versus placebo-treated patients. Further to the results of the microarray analysis, Q-RT-PCR and immunohistochemistry were also performed for validation of relevant targets.

7.2. Material & Methods

7.2.1. RNA extraction & quality assessment

Endometrial RNA was extracted as described in section 2.2.3. Prior to microarray hybridisation, the quality and integrity of each RNA sample was checked with the Agilent 2100 Bioanalyser. Approximately 500ng of total RNA was added to an RNA Nano 6000 Biochip and run according to manufacturer's instructions. This analyser provides a very sensitive qualitative analysis using a fluorescent assay, which involves electrophoretic separation for qualitative evaluation of RNA samples. As the RNA is pulsed through a microchannel over time, the amount of fluorescence is

measured and an electropherogram created by the Bioanalyser software. An RNA quality score is generated (RNA Integrity Number (RIN)). Usually, the 5s, 18s and 28s ribosomal RNA species generate three peaks in an electropherogram. With RNA degradation, these peaks become lower and background peaks appear in between. The RIN software algorithm classifies eukaryotic total RNA and determines a RIN between 1 and 10, with 1 labelling the most degraded profile (Figure 7.1.) Only samples with a RIN > 8.5 were accepted for further analysis. Prior to full array analysis, the RNA was further subjected to the Affymetrix Test3 Array, in order to identify and exclude degraded samples with insufficient target.

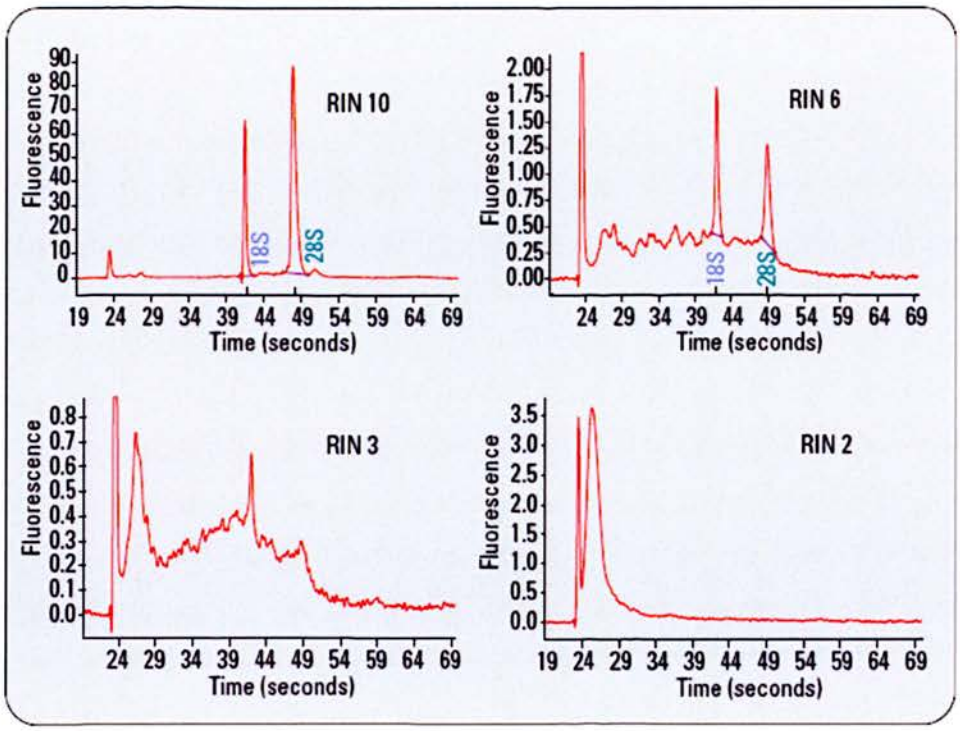


Figure 7.1. Electropherograms of RNA in different stages of degradation

High quality RNA with a high RIN of 10 generates three clear peaks in the electropherogram (5s, 18S, 28S), whilst these peaks become gradually less distinct and eventually absent depending on the stage of RNA degradation

(Derived from:

Final Report for TAP Pharmaceutical Products Inc by Forster et al, University of Edinburgh 2007)

7.2.2. Microarray

The array platform employed was Affymetrix Human Genome U133 plus 2.0, (http://www.affymetrix.com/support/technical/datasheets/human), a whole human genome expression array. This GeneChip Hybridization Array covers over 47,000 transcripts and variants, including 38,500 well-characterised human genes. It therefore offers comprehensive analysis of the transcribed human genome on a single array. It contains over 54,000 probes sets and 1,300,000 distinct oligonucleotide features, and multiple independent measurements were taken for each transcript.

7.2.3. Data processing

A standard Robust-Multiarray-Average (RMA) model was applied for between-array normalisation of microarray data. The resulting expression values were processed according to the following approaches:

7.2.3.1. Non-specific filtering

All genes were removed which never exceeded the detection threshold or never varied in expression across all samples.

7.2.3.2. Statistical hypothesis testing

For statistical analysis, a basic cell means model was applied for each gene probe signal to extract contrasts between either dose of asoprisnil (10 or 25mg) and placebo. The model was enhanced by empirical Bayes shrinkage of standard errors of genes, and significance estimates were corrected for multiple testing by the Benjamini & Yekutieli method. The genes with a high differential expression (>5-fold) between placebo and asoprisnil 10mg and / or 25mg were identified.

7.2.3.3. Pathway analysis

Differentially expressed genes with an absolute fold change of >5 and a significant corrected p-value <0.05 were subjected to Gene Ontology Enrichment analysis to determine associations with biological functions and/or diseases. DAVID (www.david.abcc.ncifcrf.gov), KEGG (www.genome.jp/kegg/pathway) and IPA (Ingenuity Inc, USA) knowledge databases and software tools were employed applying hyper-geometric test of significance for pathway membership. The genes were mapped to networks connecting different genes and proteins based on a range of interactions described in the literature. Additionally, Pearson correlation network

analysis of co-regulated genes explored how differentially regulated genes were correlated and how their concerted alteration may have an impact on biological functions.

7.2.3.4. Network clustering

Novel 3D network visualisation tools (Biolayout) enable the construction of large networks with co-expressed genes forming highly connected cliques. Hierarchical and network-based clustering algorithms are used to cluster the genes according to their expression profiles. Cohorts of genes are then identified with potentially related functionality.

7.2.4. Validation of gene targets

The effects of asoprisnil on certain gene targets, as indicated by analysis of the microarray data, were validated by taq-man Q-RT-PCR and immunohistochemistry. The genes investigated were IL-15 and CD56. As decribed below, asoprisnil appeared to have a significant effect on the expression of genes within the network associated with endometrial immune cell function. The NK cell recruiter IL-15 is central to this network and closely related to the regulation of NK cell marker CD56.

7.2.4.1. Q-RT-PCR

As described in section 2.2.3, taq-man Q-RT-PCR was applied for assessment of endometrial IL-15 and CD56 expression after administration of asoprisnil compared to placebo. For statistical analysis, ΔCT values were calculated as outlined in section 2.3.3. These were subsequently analysed by one-way ANOVA with Kruskal-Wallis testing and Dunn's multiple comparisons.

7.2.4.2. Immunohistochemistry

Immunohistochemistry was carried out for CD56, as described in section 2.2.2. Data analysis was performed as outlined in sections 2.3.1. and 2.3.2.

7.3. Results

7.3.1. Microarray analysis

28 endometrial RNA samples were of suitably high quality (RIN > 8.5; Mean RIN 9.35) to be forwarded for microarray studies. Of these, six were from placebo-treated patients, whilst 11 and 10 were from the asoprisnil 10mg and 25mg groups, respectively.

The step of non-specific filtering resulted in 26111 of 54675 probes on the chip to remain in the analysis set. Further analysis showed that 245 genes were significantly ($p \leq 0.05$ after multiple testing correction) up- or down-regulated 5-fold or greater in either or both treatment groups compared to placebo. Twice as many genes were down-regulated (166 genes) than up-regulated (79 genes). Notably down-regulated were genes associated with immune cell function including IL-15, IL-2R and granulysin, a uterine NK cell marker. The down-regulation of many genes including IL-15, IL-2R and granulysin was dose-dependent. 25mg asoprisnil had a more significant effect than the lower dose of 10mg (Table 7.1.).

	Asoprisnil 10mg	**Asoprisnil 25mg**	**Significant for both doses**
5+ fold up from placebo	30	68	19
5+ fold down from placebo	88	162	84
Total number of genes up- or down-regulated	118	230	103

Table 7.1. Differential endometrial gene expression following treatment with asoprisnil

Number of endometrial genes with statistically significant 5-fold up- or down-regulation compared to placebo using a p-value level of 0.05; twice as many genes are down-regulated as were up-regulated; the alteration in gene expression appears dose-dependent with a higher number of genes up- or down-regulated after the higher dose of 25mg asoprisnil

The analysis of differentially expressed genes for association with biological functions generated 8 significant networks (Table 7.2). Of all possible effects on the human genome, asoprisnil appeared to particularly alter the gene expression of networks associated with immune cell function. Most notable was the down-regulation of IL-15, a well-known NK-cell recruiter central to the combined networks 1 and 3 with a significant function related to the immune system (Figure 7.2.). 3D network-base clustering of the data identified several clusters of co-expressed genes, which were all down-regulated by asoprisnil. Within these clusters was a significant over-representation of genes involved in immune response, cell death and apoptosis.

7.3.2. Q-RT-PCR

Subsequent to the results of the microarray analysis, Q-RT-PCR was performed on endometrial tissue samples to evaluate the effects of asoprisnil on IL-15 and the uNK cell marker CD56. IL-15 mRNA expression was statistically significantly down-regulated by 25mg asoprisnil compared to placebo / secretory samples (Figure 7.3.). No significant difference in endometrial mRNA expression of CD56 was demonstrated between asoprisnil- and placebo-treated samples.

7.3.3. Immunohistochemistry

Immunohistochemistry demonstrated that endometrial expression of CD56 was statistically significantly down-regulated by 10 and 25mg of asoprisnil in the stromal compartment compared to placebo / secretory samples with confidence limits of –90 to –32 for 10mg asoprisnil and –94 to –33 for 25mg asoprisnil (Table 7.3.). CD56 positive cells are normally detected in the stroma of secretory endometrium, but immunoexpression was absent following treatment with asoprisnil (Figure 7.4.).

Table 7.2. Network analysis

Using network analysis, eight different networks were created, within which genes were significantly altered by asoprisnil; mostly affected are genes related to immune cell function;

most genes were down-regulated (green) and some were up-regulated (red); notably down-regulated were the uterine NK cell recruiter IL-15, IL-2R and the uterine NK cell marker granulysin (GNLY)

(Derived from:
Final Report for TAP Pharmaceutical Products Inc
by Forster et al, University of Edinburgh 2007)

	Molecules in the Network	Focus Genes	Main functions
1	ADFP, **APOD**, BEX1, CLIC6, CRABP2, CTNNB1, **DKK1**, DKK3+, DVL3, FGF19 (incl EG:9965), **GOS2**, **HSD17B2**, ID4, **IGFBP2**, **LAMB3**, LIPE, LRP5, LRP6, LRRC17, MSX2, MYF5, PAX3, PPARG, **RBP4**, SERPINAL1, **SFN+**, SFRP4, **SGK**, TCF4, TNC, **VNN1**, WNT10B, WNT3A, WNT5A, **XDH**	17	Organismal, Cellular and Skeletal & Muscular Development
2	B2M, **CLEC4E**, **CP**, **CXCL14+**, **EDNRB+**, **F3**, FGF2, FLOT1, **GOS2**, **GNLY+**, H2-Q1, H2-Q4, HLA-E, IL13, **INDO**, KLRC1, **KLRC2**, KLRC3, KLRD1, **MAOA+**, MMP17, MN1, **MT1X+**, MUC5AC, PDCD8, **PLA2G7**, PTGES, QDM, RLN2, **RXFP1+**, **SLC16A3**, **TIMP3**, TNF, VHL, YARS	16	Inflammatory Disease Respiratory disease Lipid metabolism
3	**AQP3**, CCL18, CDKN1A, CLDN2, **CTSW**, CX3CR1, CXCR3, **DPP4+**, **GZMB**, HNRPA2B1, IFNG, **IL15**, IL32, IL15RB, IL18RAP, IL1RL1, **IL2RB**, KIR2DL3, KLRB1C, **MAP3K5+**, **MT1G**, **MT1H**, **PAEP**, PDE4B, PLEK, **PRF1**, PRG1 (incl EG:5552), **RARRES1+**, S100A11, SERPINB9, SFRP1+, **SLC3A1**, SPDEF, SPINK7, **WNT4**	16	Cell Death **Immune response** Cancer
4	CARD10, **GZMA**, **GZMB**, IHH, IKBKG, IL2, IL25, IL17RB, **KCNQ1**, KLF11 (incl EG:8462), LAMA1, **LAMA2+**, LAMA3, LAMB1, **LAMB3**, LAMB1-2, LAMC1, LAMC2, **LAMC3**, LIF, MALT1, MAPK1, MET, MGC45438, NEFH, PDE4B, POMC, PPP2CB, PPP2R2A, PPP2R2C+, **SNF1LK**, **SOD2+**, **SULF1**, TFF3, TRAF6	16	Dermatological Diseases Genetic Disorder Cellular Movement

	Molecules in the Network	Focus Genes	Main functions
5	ACP5, CCNG2, **CD36+**, CITED2, **COMP**, CPB2, DAPK1, **FKBP5+**, FOXG1B, **FOXO1A+**, HNRPU, HOXA9, HOXD3, HTRA1, **IGFBP2**, ITGB6, JAG1, LPA, LTBP1+, MMP11, **MT2A**, NLK, **PDZK1IP1**, ROCK1, SERPINA1, **SGK**, **SPP1**, STARD10, TAF4B, TBX21, TBXA2R, **TCN1**, TGFB1, **TGM2**, TNFRSF11B	15	Cellular Growth and Proliferation Cancer Tumour Morphology
6	**ACSL5**, AGRP, ANK2, CART, CD247, CD40LG, DLG3, DUSP5, **G0S2**, **GDA**, IFITM1, **INDO**, INHBA, ITPR1, KCNK3, KIR3DL1, **LEFTY1**, LEP, **MT1E**, **MTHFD2**, NODAL, PKMYT1, PRKCA, SCN3A, **SLC15A1**, **SLC1A1+**, SLC8A1, SNAP25, SNAPAP, STXBP6 (incl EG:29091), TNF, **TRA@+**, TRAT1, TRD@, TRH	13	Cell-to-Cell Signalling and Interaction **Immune Response** Cancer
7	**ACSL4**, ACTA2, ACTB, AGC1, BCL2L11, BIRC5, CCNB1, CD9, CDC25A, CDH1, CDK6, **DPP4+**, DPT, E2F1, ERBB2, **FBLN2**, HMGB2, IGSF4 (incl EG:23705), ILK, **KRT7**, MAP3K14, MUC1, **NDRG1**, NFYB, NOPE, **PLCL1**, PVRL3, RRM1, **RRM2**, **S100P**, SMARCB1, TFF3, **TIMP3**, TOP2A, VIL2	13	Cancer Haematological Disease Cellular Growth and Proliferation
8	**ALDH1A3**, ANPEP, **AOX1**, **BASP1**, BTBD11, CD83, CHAT, CHGB, **CLEC4E**, **CYP3A5+**, E2F5, EFEMP1, **ID2**, IL6, IL15RA, KRAS, MYOP1, **NFIL3**, NGFB, NR1I2, NTRK2, **PAX8**, PDIA3, POSTN+, RGS20, **SNX10**, STMN2+, TCF12, TCF23, TERT, TNFRSF11B, TNFSF11, TOP2A, WNT5A, XBP1	12	Cellular Development Cancer Cell Death

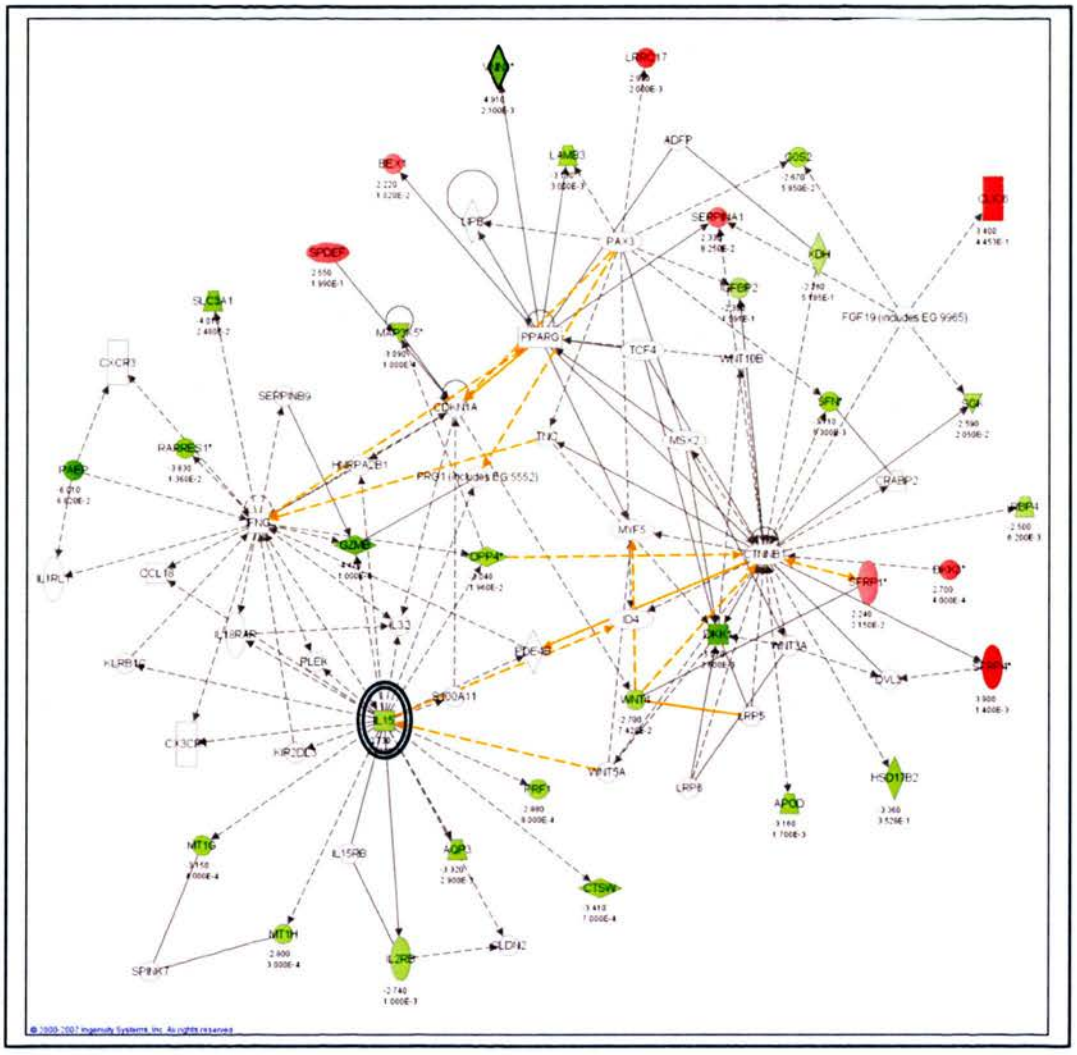


Figure 7.2. Gene Network Analysis with differentially expressed genes

Alterations of gene expressions by administration of asoprisnil within biological networks associated with immune cell function and apoptosis (networks 1 and 3); most genes within these networks are significantly down-regulated (green), only few are up-regulated (red); central to the network and notably down-regulated is the uNK cell recruiter IL-15 (circled)

(Derived from:

Final Report for TAP Pharmaceutical Products Inc by Forster et al, University of Edinburgh 2007)

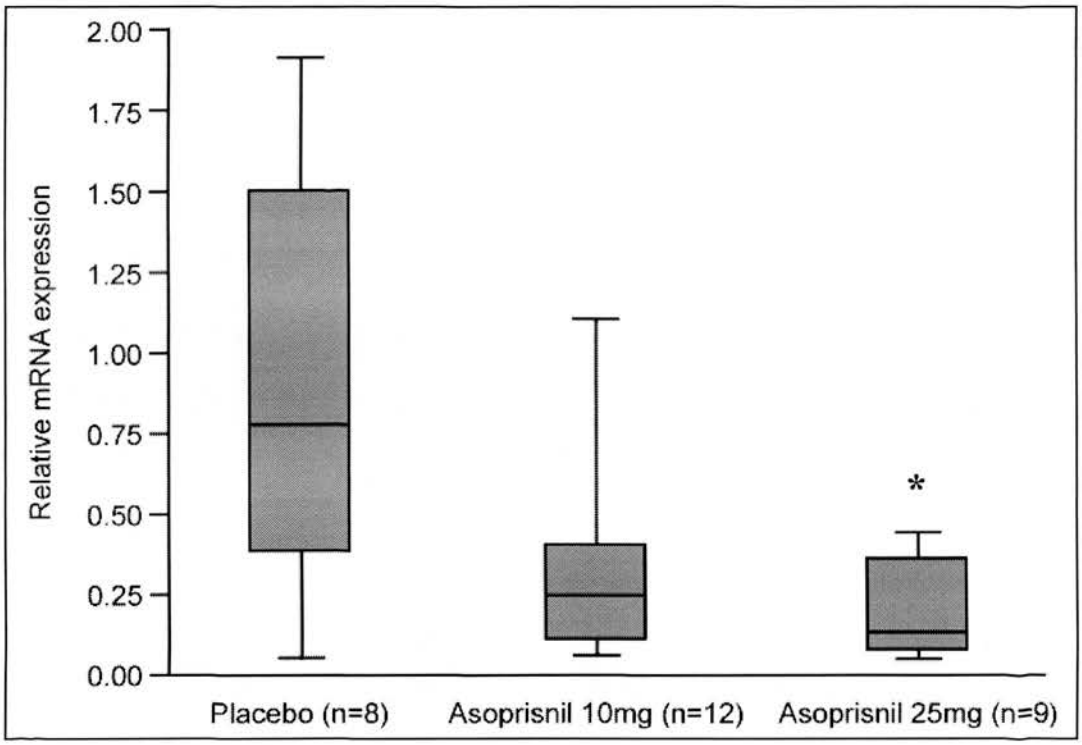


Figure 7.3. IL-15 mRNA expression

IL-15 mRNA expression is statistically significantly down-regulated by 25mg asoprisnil (* = $P<0.05$) compared to placebo / secretory samples;
in the box plots, the box spans between lower and upper quartiles with the horizontal line within it representing the median; the whiskers extend to the minimum and maximum observations; each asoprisnil group is compared to placebo / secretory phase using Dunn's multiple comparison test

CD56 - Histoscore -	Treatment Group			
	Placebo / Proliferative (N=2)	Placebo / Secretory (N=8)	Asoprisnil 10 mg (N=12)	Asoprisnil 25 mg (N=11)
STROMA				
Mean +/- SD	40 +/- 0	69.4 +/- 48.36	8.3 +/- 12.31	5.9 +/- 9.7
Minimum - Maximum	40	0 – 135	0 – 35	0 – 30
Median	40	77.5	0	0
95% Confidence intervals (CI); Mean difference to placebo	N/A	N/A	-90 to -32.2	-94.4 to -32.5
P-value[a]	N/A	N/A	0.004[$]	0.004[$]
PERIVASCULAR CELLS				
Mean +/- SD	7.5 +/- 10.61	41.9 +/- 38.7	1.3 +/- 4.33	4.1 +/- 8.31
Minimum - Maximum	0 – 15	0 – 90	0 – 15	0 - 25
Median	7.5	27.5	0	0
P-value[a]	N/A	N/A	0.001[$]	0.011[$]

Table 7.3. CD56 immunohistochemistry

Histoscores of endometrial CD56 expression comparing placebo-treated samples (secretory phase) to asoprisnil-treated samples with highly significant p-values; CD56 expression is completely absent after treatment with asoprisnil

[a] Each asoprisnil group is compared to placebo / secretory using Wilcoxon's rank sum test

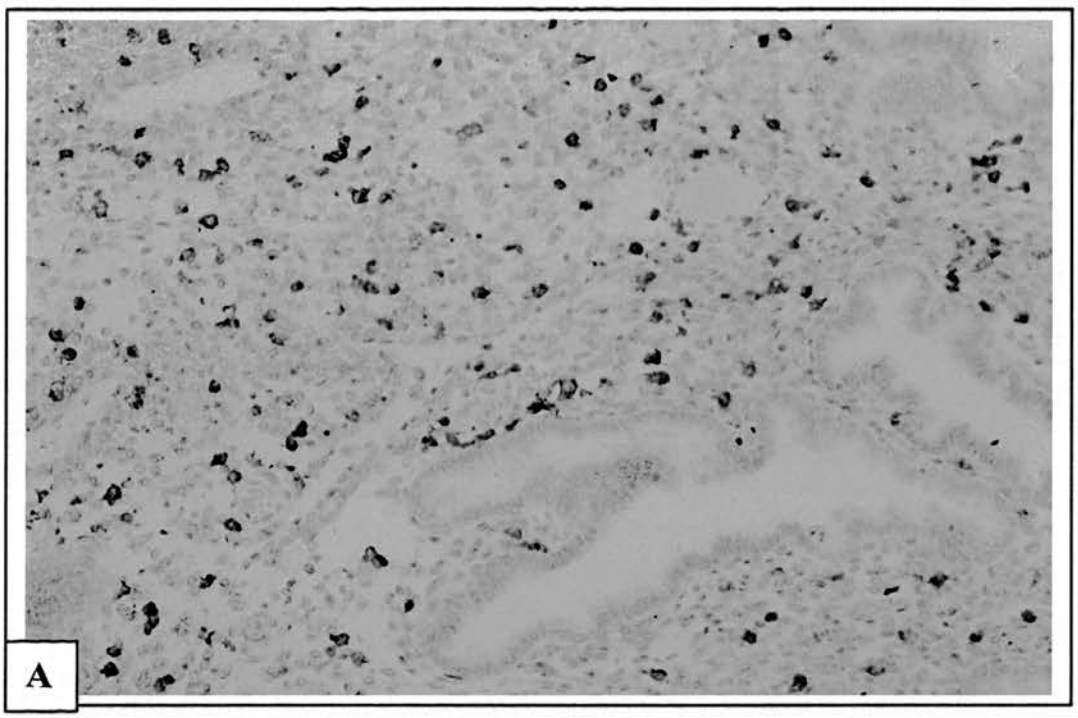
[$] Denotes statistical significance at 0.05 level using Hochberg's multiple comparison procedure

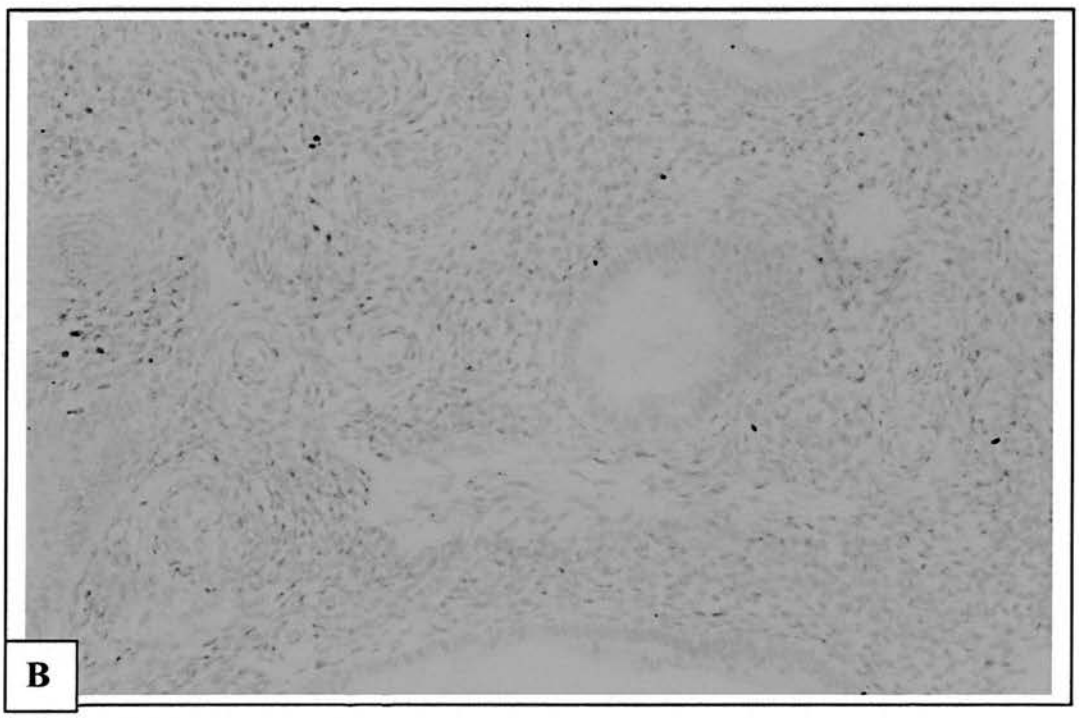
N/A = not applicable

Figure 7.4. CD56 immunohistochemistry

A Endometrial CD56 immunoexpression in placebo-treated samples (secretory phase)

B Endometrial CD56 immunoexpression is absent following treatment with asoprisnil


A


B

7.4. Discussion

Analysis of whole human genome expression array data and endometrial gene expression profiles following treatment with asoprisnil showed a down-regulation of genes associated with local immune cell function and apoptosis. The microarray data specifically revealed a dose-dependent down-regulation of the uterine natural killer (uNK) cell recruiter IL-15 and the uNK cell marker granulysin by asoprisnil. Consistent with these findings, subsequent Q-RT-PCR also showed a down-regulation of IL-15. Immunohistochemistry demonstrated an absence of CD56 (uNK cell marker) positive cells in endometrial stroma of women administered 10 or 25mg asoprisnil daily for 12 weeks. These results indicate that asoprisnil exerts a significant effect on local immune responses, which may play a role in remodelling the endometrial vasculature.

Endometrial mRNA expression of CD56 appeared unaltered by administration of asoprisnil. This discrepancy may be explained by the relatively short sequence of the primer used in the Q-RT-PCR analysis. Whilst the whole genome is analysed in the microarray, the Q-RT-PCR focuses on a short sequence of the relevant gene only and may not always detect alterations, which are not reflected in that particular sequence.

A role for immune cells in spiral arteriole remodelling has previously been demonstrated in the early stages of human pregnancy[225]. IL-15 is known to influence the process of endometrial vascular remodelling via its role in post-ovulatory recruitment, differentiation and proliferation of uNK cells[226,227]. In the secretory phase, 70-80% of endometrial lymphocytes are uNK cells, which display a unique phenotype ($CD56^{bright}$, $CD16^{dim}$, $CD3^{dim}$) and differ from peripheral blood NK cells ($CD56^{dim}$, $CD16^{dim}$, $CD3^{-}$). Across the menstrual cycle, endometrial uNK cell numbers vary in parallel with IL-15 expression[43]. Endometrial uNK cells appear to influence the development of decidual spiral arteries, as they produce interferon (IFNγ) and other angiogenic factors, which possibly regulate the gene α2-macroglobulin (α2M)[228].

IL-15 has been demonstrated to have a central role in attracting peripheral blood NK cells into the endometrium during the secretory cycle phase[226]. Endometrial IL-15 expression is up-regulated in the luteal phase in endometrial stroma and notably in the perivascular cells surrounding the spiral arteries[42,43]. The complex process of IL-15 regulation in human endometrial cells has also been studied in vitro[229]. IL-15 could be stimulated by prostaglandin E_2 (PGE_2) and 8-Bromo cAMP in the presence of interferon-γ (IFN-γ) in human endometrial stromal cell cultures. The addition of

progestins appeared to enhance these responses[230]. It has been suggested that ovarian steroids regulate IL-15 expression, but this control may be indirect and also influenced by other cytokines with immune regulatory functions such as tumour necrosis factor (TNF)-α and transforming growth factor (TGF)-β[231].

The variance in endometrial IL-15 expression corresponds to the fluctuation of uNK cells[43]. Experiments with IL-15 knockout mice substantiate the hypothesis of a close link between IL-15 and uNK cell differentiation, proliferation and function[227,232]. Mice lacking a functional IL-15 are deficient in uNK cells with a complete absence in their implantation sites. Morphologically, these IL-15 -/- mice show decidual pathologies with thick-walled arteries with narrowed lumen and swollen endothelial cells and acellular, oedematous decidua[227]. Another study equally demonstrated that spiral artery modification was absent, and there was a lack of decidual integrity compared to normal mice[232].

The functions of uNK cells have been studied in mouse models and particularly in early pregnancy[228,233]. uNK cells produce cytokines and angiogenic growth factors affecting the decidual vasculature[234]. uNK cell-derived IFN-γ is essential in triggering spiral artery modification in early pregnancy[233]. IFN-γ induces gene transcriptional alterations and has been demonstrated to up-regulate the α2M gene family[228]. Both experiments with mice deficient in uNK cells and mice deficient in members of the α2M family show a significant effect on the vasculature in implantation sites[228,235]. The decidual vessels in immunodeficient mice lacking uNK cells are characterized by an unusual morphology with elevated wall to lumen ratios and endothelial hypertrophy[236]. The larger spiral arterioles appear thick-walled with a deficit in elongation and coiling. Interestingly, the morphology of these spiral arteries in uNK cell deficient mice is reminiscent of the spiral artery morphology observed in asoprisnil-treated human endometrium[227].

The precise role of the uNK cell in human endometrium is still to be clearly defined, and most studies have been done in the context of the development of decidua in early pregnancy. uNK cells are the predominant leucocytes in the early pregnancy decidua, and studies in women with recurrent early pregnancy loss implicated an association between alterations in uNK cell populations and early pregnancy failure[237]. Very little is known about the significance of uNK cells outwith the context of implantation and early pregnancy. It has been suggested that endometrial NK cells have no significant role prior to conception[238]. However, it has also been recognised that the menstrual process is influenced and regulated by inflammatory mediators[33]. A large influx of leukocytes and particularly an increase in uNK cells has been demonstrated in the human endometrium in the late secretory phase[19], and

the role of the perivascular cell in this inflammatory response has been highlighted[9]. A study of endometrium from women with irregular bleeding in the context of hormone replacement therapy (HRT) found uNK cell numbers and endometrial IL-15 expression to be increased during bleeding episodes compared to HRT users with no bleeding[239]. Similarly, endometrial immunoexpression of IL-15 has been found elevated in women with irregular bleeding whilst using levonorgestrel implants compared to those with regular cycles or amenorrhoea[240]. These data suggest an association and, considering the known link between uNK cells and endometrial vascular remodelling, possibly a causative relation between uNK cells and uterine bleeding pattern.

In this study, a whole human genome expression array was used. This approach had the advantage of being completely unbiased and allowing major alterations on a genome wide level to be detected. Analysis of the effect of asoprisnil on endometrial gene expression profiles has resulted in the observation that the main genes altered by asoprisnil treatment compared to placebo are related to immune response. The results indicate a down-regulation of several key factors within the network affecting the local immune system, central to which is IL-15. Down-regulation of IL-15 was confirmed on mRNA level. Local effects of IL-15 are thought to be mediated via activation of uNK cells. uNK cells have been demonstrated to be absent in endometrium with asoprisnil treatment (as indicated by their marker CD56). These observations support the previously formulated hypothesis that the clinical effects seen with asoprisnil are mediated by a directly modulating effect on endometrial vasculature, possibly via the suppression of local immune responses, IL-15 and subsequently uNK cells.

7.4.1. Limitations of the study

The results of the microarray studies have prompted the development of a whole new concept of the mechanism of action of asoprisnil. It appears to comply with previous suggestions that asoprisnil may exert a direct effect on the endometrial vasculature. However, the indicated modulation of local immune cell function represents a novel approach to understanding the effects of asoprisnil on endometrium.

This concept needs to be consolidated by specifically analysing multiple relevant genes and proteins. In this study, the effects on IL-15 mRNA and CD56 protein have been investigated. Difficulties with obtaining IL-15 antibodies for immunohistochemistry have prevented analysis of the effects on IL-15 on protein level. The striking suppression of CD56 protein could not be confirmed on mRNA

level, possibly due to the limited sequence of the primer used for Q-RT-PCR analysis. In order to further develop and confound the concept of asoprisnil modulating endometrial immune cell function, a wider variety of target genes and proteins will need to be studied.

7.5. Conclusion

Asoprisnil down-regulates endometrial genes known to be involved in local immune cell function and apoptosis, in particular IL-15. Asoprisnil also down-regulates the uNK cell marker protein CD56 in endometrial stroma and perivascular cells. The effects of asoprisnil on local immune responses may play an important role in morphological and functional changes in endometrial vasculature. These may contribute to the clinical effect of suppressed uterine bleeding.

CHAPTER 8

FINAL DISCUSSION

8.1. Synopsis of results

This thesis has evaluated the effects of the PRM asoprisnil in women with symptomatic uterine fibroids. Primarily, clinical efficacy and tolerability were established. Particular emphasis of further studies was placed on assessment of endometrial safety and on the investigation of the mechanism of action.

As consistently demonstrated with PRMs in humans and non-human primates, this study consolidated the finding of a remarkable effect of asoprisnil on menstrual bleeding pattern with profound suppression and minimal breakthrough bleeding. Even in this group of women who had mostly presented with very heavy menstrual bleeding, including some with blood loss of over 200ml per period, the higher dose of 25mg asoprisnil caused amenorrhoea in over 90%. There was also a favourable influence of asoprisnil on the quality of life of these women, who had consented to major abdominal surgery due to the impact of their symptoms on their quality of life. Clinically, in this study asoprisnil appeared highly effective and also had a favourable safety and tolerability profile. In keeping with previous studies, these significant clinical effects were achieved with mostly continued follicular ovarian activity and therefore maintained serum E levels. Doppler sonographic assessments of uterine artery blood flow demonstrated a moderate reduction indicating that a vascular effect may be an important contributor to the mechanism of action of asoprisnil.

Thorough assessment of endometrial morphology following administration of asoprisnil demonstrated a unique pattern, which was inconsistent with previously known histological classifications. Particularly notable were changes in glandular and vascular appearances. Cystic glandular dilatation, a feature previously found following administration of mifepristone, was demonstrated in the asoprisnil treated samples. However, this was not associated with any hyperplastic changes, and specifically the mitotic counts were very low. Further investigations of proliferation marker expression revealed no evidence of asoprisnil inducing endometrial proliferation. On the contrary, there was significant suppression of proliferation marker expression in the stroma. The results of this study indicate that administration of asoprisnil for 12 weeks does not increase the risk of endometrial hyperplasia or cytological atypia. This conclusion was further supported, when the expression of the tumour suppressor gene PTEN was assessed and found to be unaltered by asoprisnil.

Morphologically prominent were the changes of the endometrial vasculature in the asoprisnil treated samples. These were specific to the endometrium and consisted of

an increased occurrence of both aggregates of thin-walled vessels and clusters of thick-walled muscularized spiral arterioles compared to controls. These distinct features further indicated that vascular remodelling might play an important role in mediating the effects of asoprisnil.

As asoprisnil ligates to a sex steroid receptor, the response of endometrial sex steroid receptor expression to asoprisnil was studied on both mRNA and protein levels. Expression of ERα, PR and AR were significantly altered. Notably there was a differential effect in endometrial stroma and epithelium. In particular, PR was up-regulated in the epithelium but down-regulated in the stroma. Expression of PR was specifically decreased in perivascular cells. This may be significant for the modulation of endometrial vascular function by asoprisnil. There was also significant up-regulation of ERα and AR. AR expression was specifically increased in the stroma. Similar effects of PRMs on AR have previously been demonstrated and hypothesized as significant for the endometrial antiproliferative effect.

To elucidate the mechanism of action of asoprisnil further, endometrial RNA samples were submitted for microarray analysis and evaluation of endometrial gene expression profiles following administration of asoprisnil compared to controls. With the unbiased approach of using a whole human genome expression array, the results indicated a notable down-regulation of genes related to the local immune system. Specifically, IL-15 expression was suppressed, which was confirmed at the mRNA level. Due to previous evidence that local effects of IL-15 are mediated via activation of uNK cells, the expression of uNK cell marker CD56 was further investigated and found to be absent in asoprisnil treated endometrial stroma and perivascular cells. These findings strongly support the previously formulated hypothesis that asoprisnil exerts its effects by direct modulation of the endometrial vasculature, possibly via suppression of local immune responses.

8.2. PRMs in clinical practice

Since the first discovery of mifepristone in 1981, many studies have been conducted to evaluate the clinical applicability of various PR ligands. Two compounds have been licensed in the context of fertility control but in addition, their usefulness has been proposed predominantly for management of benign gynaecological conditions. The main property rendering PRMs such suitable agents is the endometrial antiproliferative effect with associated suppression of menstrual bleeding. This study confirmed that administration of asoprisnil for 12 weeks appears clinically effective with profound reduction of uterine blood loss. With further improvements in quality

of life and the favourable tolerability profile, it may be concluded that PRMs, and in particular asoprisnil, offer themselves as an alternative for the medical management of gynaecological entities associated specifically with heavy uterine bleeding.

8.3. PRMs and endometrial safety

The property of PRMs and specifically their PR antagonistic effect may and did give rise to concerns regarding the exposure of the endometrium to unopposed oestrogens and hence endometrial safety. Early clinical studies with mifepristone fuelled these concerns as cases of endometrial hyperplasia were reported. Subsequent detailed morphological studies have however indicated that the main feature previously leading to the classification of hyperplasia was cystic glandular dilatation and did actually occur in isolation without any other hyperplastic changes. It has since been appreciated that PRMs induce a unique class effect in the endometrium. The "Dictionary of Endometrial Biopsy Diagnoses for Clinical Trials with SPRMs" was developed by a group of expert gynaecological pathologists in cooperation with TAP Pharmaceuticals Inc. and Diagnostic Cytology Laboratories in the USA to classify these characteristic endometrial appearances. No cases of hyperplasia or cytological atypia have been described with asoprisnil. The results of this study supported the conclusion that administration of asoprisnil for 12 weeks is safe, as there was no evidence of increased proliferation and indeed significant suppression of proliferation marker expression in the stroma. Maintained PTEN expression provided further reassurance that asoprisnil does not induce endometrial carcinogenesis.

8.4. The mechanism of action of asoprisnil

The endometrial antiproliferative effect of PRMs and PAs became apparent early in the course of their discovery and was unexpected. The mechanism of action has still not been fully elucidated. As it occurs with maintained serum E levels and with administration of compounds with predominantly P antagonistic properties, it is not immediately obvious how this antiproliferative effect may be achieved. The up-regulation of stromal AR expression demonstrated in this study has previously been highlighted as potentially significant in this context. It has also been hypothesized that suppression of endometrial proliferation may be mediated via a direct effect on the endometrial vasculature. This hypothesis was supported by several findings in this study. Uterine artery blood flow was found to be moderately reduced by asoprisnil compared to controls. Morphologically, there were striking changes to the

endometrial vessels and specifically the spiral arterioles. Most significant were the results of the whole human genome expression array analysis. Of all possible effects on endometrial gene expression, the genes related to local immune cell function were significantly down-regulated by asoprisnil. Specifically IL-15 expression was altered, which is known to affect endometrial vascular remodeling via regulation of uNK cells. These findings were a strong indication that asoprisnil modulates the local immune response system, which may alter the function of the endometrial vasculature. It appeared that these effects of asoprisnil might be pivotal in the understanding of its mechanism of action.

8.5. Suggestions for future studies

With evidence for clinical efficacy and endometrial safety of asoprisnil administered at doses of 10 or 25mg for 12 weeks, future studies will be required to establish that long-term administration will fulfill the same criteria. It will be of particular interest to investigate how endometrial morphology develops with continued exposure to asoprisnil. Specific features to observe would be the glandular architecture and the endometrial vasculature. It will be important to establish whether the cystically dilated glands found in asoprisnil-treated samples after 12 weeks, remain at a similar appearance, dilate further or become atrophic. It may also be an objective to study whether the addition of another PR ligand or potentially a small dose of progesterone alters or even abolishes the occurrence of these dilated glands. Of specific interest will be the development of the endometrial vessels and the observed aggregates of thick-walled muscularized spiral arterioles over time, particularly in view of their proposed implications for the mechanism of action of this class of compound.
The most profound clinical effect of asoprisnil is the suppression of uterine bleeding. Currently, the most efficient non-surgical management option for heavy menstrual bleeding (HMB) is the levonorgestrel containing intrauterine system (LNG-IUS Mirena®). In a phase III trial, the effects of asoprisnil on HMB may be compared to the effects of the LNG-IUS. It would be interesting to assess and compare the suppression of menstrual bleeding, but also the occurrence of unscheduled break-through bleeding. The latter is a common reason for discontinuing treatment with the LNG-IUS but has not been reported with asoprisnil. A phase III trial could be conducted with oral administration of 25mg asoprisnil, which has been demonstrated to have a more profound effect than 10mg without increase in side effects in this study. Alternatively, an intrauterine system containing asoprisnil may be developed for a trial comparing its effect to the LNG-IUS. It will be important to gather

evidence for endometrial safety with any future clinical trial and particularly with prolonged periods of treatment. It may be proposed that an endometrial biopsy is obtained at 3-6 monthly intervals for this purpopse.

The findings of the microarray studies have unveiled new insights into potential pathways of the effects of sex steroid receptor ligands. The relevance of the local immune system for endometrial vascular remodeling and the evidence for its modulation by asoprisnil enables a whole new approach into the study of these compounds and the study of endometrial function in general. Further studies will need to elucidate the exact components of these pathways and will then allow to determine ways of modulation for therapeutic purposes. The role of the endometrial immune response may be significant not only for fertility treatment but also for the management of common symptoms such as heavy menstrual bleeding and dysmenorrhoea, and related benign gynaecological conditions such as uterine fibroids and endometriosis.

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APPENDIX I

PATIENT INFORMATION SHEET

Ethics Submission No.: 386/02/w

Patient Information Sheet

Full Title: **Study C02-003: "A Phase II, 12-Week, Double-Blind, Placebo Controlled Study to Evaluate the Effects of J867 on Uterine Artery Blood Flow and the Morphology of the Endometrium, Myometrium and Uterine Leiomyomata in Subjects with Uterine Leiomyomata Scheduled for Hysterectomy"**

Short Title: **"J867 in women with uterine fibroids"**

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask the research doctor or nurse if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?

Clinical research is performed in patients to see whether or not a new treatment works and is safe to use.

The purpose of this study is to determine the effect of two doses of the investigational medication asoprisnil on the uterus (womb) compared to placebo (dummy treatment) when given daily for 12 weeks to patients with uterine fibroids. The effects of asoprisnil on blood flow in the uterus and on the layers of the uterine wall will be studied. Your participation in this study will include a screening period of up to six weeks, medication period for 12 weeks, followed by your scheduled hysterectomy, and a 6-week follow-up period after hysterectomy. The study will involve at least 5 visits to the clinic prior to hysterectomy and 1 visit to the clinic following hysterectomy (a minimum of 7 visits, including surgery).

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Why have I been chosen?

You have been chosen to take part in the study because you have been diagnosed with uterine fibroids and the doctor has decided that you require a hysterectomy.

There will be approximately 45 patients enrolled in this study at 4-5 centres in the UK.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. However, you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

In order to take part in the study, you must be scheduled for a hysterectomy at the end of the study treatment period due to symptoms associated with uterine fibroids. Participation in the study involves 4-5 visits over a 3-month period prior to your hysterectomy plus a follow-up visit 6 weeks after surgery.

Study Procedures:

1st Visit - **Screening** (performed within 45 days of starting the study medication)

The research doctor or staff will ask you questions to determine if you are eligible to participate in this study. You will be asked about any medication you have taken in the last month.

You will also be asked to have:

- Complete physical, breast and pelvic examinations.
- Vital signs (measurement of heart rate, blood pressure, and body temperature) and measurements of height and weight.
- Cervical smear.
- Endometrial biopsy - The endometrial biopsy is a test that involves inserting a small tube into the neck of the womb to obtain small pieces of tissue. If difficulty is encountered in passing this tube into the uterine opening, another small tube may also need to be inserted to help widen the opening. This is a routine procedure conducted in the outpatient setting to investigate menstrual problems.
- Electrocardiogram (ECG) - measures the electrical activity of your heart
- Blood and urine sample collection for routine laboratory tests (chemistry, haematology, coagulation, lipids, hormones, and urinalysis), and pregnancy testing.
- Contraception counselling.
- Transvaginal ultrasound (TVU) procedure(s) - The TVU is a pelvic ultrasound examination involving the insertion of a probe instrument into the vagina to produce a picture of the pelvic area using sound waves and is done to

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determine the thickness of the lining of the uterus and the size of the uterus and fibroids.

- Interview by the research staff regarding your medical, fibroid, gynaecologic, menstrual, medication (including other hormonal preparations), and social (smoking, caffeine and alcohol consumption) histories will be conducted and recorded.
- Bleeding Diary – you will receive a diary to record daily uterine bleeding for a minimum of one full menstrual cycle prior to your first tablet (Day 1) as well as any non-steroidal anti-inflammatory (NSAIDs) medications and transexamic acids taken. You will be given sanitary protection products for use in order to maintain consistency across the study.
- Urine Collections – you will be asked to collect urine samples twice a week (Monday and Thursday or Tuesday and Friday) starting on one of the first 5 days of your next menstrual cycle. The first urine sample of the day should be collected. The samples will be used to measure your ovarian hormone levels throughout the pre-treatment and treatment periods. Specific collection containers will be provided. The date of collection should be marked on the containers and the samples should be stored at –20 ° C in the freezer until you bring them to the clinic at your next visit.

2nd Visit – **Pre-dose (day 20-23 of menstrual cycle prior to dosing)**

The following procedure will be performed during days 20 to 23 of your menstrual cycle immediately prior to dosing:

- Transvaginal ultrasound (TVU) including measurement of blood flow in the uterus

3rd Visit – **Pre-dose (Day –1)**

The following procedures may be performed at the 2nd Visit or on the day before your first dose of the study drug:

- Complete physical andbreastexaminations
- Vital sign and weight measurements
- Blood and urine sample collection for routine laboratory tests (chemistry haematology, coagulation, lipids, hormones, and urinalysis)

The following procedures will be performed the day before your first dose of the study medication:

- Blood and urine sample collection for pregnancy testing
- Contraception counselling
- Symptom assessment – your symptoms since the last clinic visit will be assessed
- Uterine fibroid symptom and health-related quality life questionnaire (UFS-QOL)
- Review of the bleeding diary from the screening period
- Return of urine collection samples
- Interview regarding use of other medications
- Dispensing of new diaries, sanitary protection products, urine collection tubes, and study drug (8-week supplies)

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Study Treatment

The study is "blinded" which means that neither you nor your doctor will know what treatment you receive until after the study is completed. Sometimes, because we do not know which way of treating patients is best, we need to make comparisons. Patients in this study will therefore be put into groups and then compared. The groups are selected by a computer list, which has no information about the individual – i.e. by random chance (like the flip of a coin) and will determine which of the three treatments you will receive. In an emergency situation we are able to find out which treatment you are on. There is an equal chance of receiving any one of the 3 treatments. If you qualify to take part in this study, you will receive one of the following treatments:

- 10 mg asoprisnil or
- 25 mg asoprisnil or
- Placebo for asoprisnil

A **placebo** is a dummy treatment, which looks like the real thing but contains no active ingredients.

During the treatment phase you will be required to keep a daily record of any vaginal bleeding and medication use in the diary provided to you. The diary should be completed before going to bed each night so that information for the entire day may be captured.
At each visit you will be supplied with new diaries, sanitary protection products (in order to ensure consistency throughout the study), urine collection tubes, and study medication supplies.

A member of the study staff will call you at **Week 4** to discuss the study medication and other medications you may have taken since the Day –1 visit and any side effects you may have experienced during that period. You will also be counselled on the use of contraception.

4th Visit

At the **Week 8 Visit**, the following procedures will be performed: brief physical examination; vital sign and weight measurements; blood and urine sample collection for routine laboratory tests and pregnancy testing; contraception counselling; symptoms assessment; review of the bleeding diary from the previous visit; and an interview regarding use of other medications and any medical problems. You will be asked to return the urine samples that you collected during the previous 8 weeks at this visit. A new diary, sanitary protection products, urine collection tubes, and a 4-week supply of study medication will be dispensed.

5th Visit

At the **Week 12/Final Treatment Visit**, the following procedures will be performed: vital signs and weight measurements; complete physical, breast, and pelvic examinations; cervical smear; electrocardiogram (ECG); transvaginal ultrasound (TVU) including measurement of blood flow in the uterus; blood and urine sample collection for routine laboratory tests and pregnancy testing; contraception counselling; symptom assessment; Uterine fibroid symptom and health-related

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quality of life questionnaire (UFS-QOL) review of the bleeding diary from the previous visit; and an interview regarding use of other medications and any medical problems. Your pelvic examination and Pap smear may be performed at the Hysterectomy Visit instead of this visit. You will be asked to return the urine samples that you collected during the previous 4 weeks at this visit.

At the 4th (week 8) and 5th (week 12) visits, a member of the study staff will evaluate how well you are taking your study medication by counting the number of tablets in the blister card that you will bring back at each clinic visit.

You will be asked to have urine and blood collected at all study visits for laboratory testing including a urine pregnancy test. If at any time you have a positive pregnancy test, you will be withdrawn from the study. Approximately 2 to 3 tablespoons of blood will be collected at each clinic visit. At week 8, an additional ½ to 1 tablespoon of blood will be drawn for extra hormone tests.

In addition, some procedures may be performed at other times during the study if medically necessary (for example, if the cervical smear or endometrial biopsy sample was not adequate).

Some of these procedures might be done anyway as part of your standard care even if you do not take part in this research study. The study doctor or a member of the study staff can answer any questions you may have about the procedures that are not part of your standard care.

Hysterectomy Visit:

Hysterectomy will be performed within 24 hours after the Week 12 Visit (4th – 5th visit). Prior to hysterectomy, blood will be taken for the measurement of hormone concentrations. If you did not have your pelvic examination and Pap Smear at the previous visit (5th visit), they will be performed at this visit prior to the hysterectomy. After your uterus has been removed, biopsies (a small section is cut away) of all layers of the uterine wall will be taken.

If your surgery is delayed by more than 24 hours, you will have enough medication to continue treatment for up to 4 additional weeks. In that case, the Week 12 ultrasound scan will be performed within 24 hours prior to your rescheduled hysterectomy. Blood will be taken for the measurement of hormones on the day of the rescheduled hysterectomy.

You will be asked to give permission for storage of the hysterectomy sample for use in future research studies on uterine function. The precise nature of some of these studies will depend upon future scientific advances. Some of the potential future projects may be carried out by researchers other than your own study physician. Tissue taken for study assessments will be stored at the University of Edinburgh.

6-Week Follow-Up Visit:

You will be asked to return to the clinic 6 weeks after your hysterectomy for complete physical, breast, and pelvic examinations and an interview regarding any medical problems. In addition, if you did not have your ovaries removed during hysterectomy, you will have an examination of the ovaries by transvaginal ultrasound.

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If you decide to take part in this research study, you will be asked to complete the study visits and other procedures and to follow the instructions of the study doctor and study staff. If you decide to stop participating in the study or if you do not follow instructions, it is important that you notify the study doctor or study staff to help you make these decisions safely. If you stop participating, you will also be asked to see your study doctor to have the appropriate procedures done at your final study visit. It will be important for you to agree to an endometrial biopsy, as an outpatient procedure (as described earlier on page 2) for a safety assessment in the event of early discontinuation from the study.

Your participation in this study may also be discontinued without your consent by the doctor conducting the study or by the sponsoring agency at any time for reasons such as: if it appears that participation in the study may be medically harmful to you, if you fail to follow directions for participating in the study, if it is discovered that you do not meet the study requirements, or if the study is cancelled. You will be made aware of any significant new findings that may affect your decision to remain in the study.

What do I have to do?

Unless you have been sterilised or your partner has had a vasectomy, you must agree to use a medically proven double-barrier method of birth control (condoms with spermicide, sponge with spermicide, or diaphragm with spermicide) during the study and until you have had your hysterectomy or until your first menses after treatment if you decline surgery.

If you think you may be pregnant, you should immediately stop taking the study medication and contact your study doctor. If you become pregnant you will be withdrawn from the study, and the study doctor will track your pregnancy and report the outcome to the sponsor and the Ethics Committee at the hospital.

Hormonal products (including oral birth control pills or DepoProvera®) plus some other medications (prescription medications, over-the-counter medications, and nutritional supplements) that you may be taking at the present time must be discussed with your research doctor who will have a list of excluded medications. You will be asked to stop these medications before starting the study medication. This is called a washout period where the effects of your current medications leave your body. The length of the washout period depends upon which medications you are using.

Because of the possible interactions of asoprisnil with other medications or foods, you will be asked to tell your study doctor all the medications (prescription medications, over-the-counter medications, and nutritional supplements) that you may be taking, and you will need to receive approval for their use during the study.

Grapefruit may not be eaten and grapefruit juice may not be consumed during the medication period of the study since it may affect how your body uses the study medication. There are no other dietary restrictions imposed on you. Your general practitioner will also be notified of these restrictions and your participation in the study.

If you have any questions please discuss them with your research doctor.

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What is the medication or procedure that is being tested?

Asoprisnil is a research medication that modifies the effects of the female hormone progesterone. It is currently under development by TAP Pharmaceutical Products Inc. for the treatment of patients with gynaecological disorders such as endometriosis and fibroids. More than 300 healthy females have received asoprisnil in clinical studies performed to date. If you are entered into the study, you will be given an emergency card, which contains brief details regarding your treatment. Your research doctor will provide you with the card. This card should be carried around with you at all times and shown to anyone who treats you e.g. doctor, nurse, dentist or pharmacist. For this study, you are required to take one tablet (study medication) orally (by mouth) at the same time every morning for 12 weeks.

What are the alternatives for diagnosis or treatment?

Participation in this study is not a substitute for your usual ongoing medical care by your regular doctor or specialist. You do not have to participate in this study to receive treatment for your fibroids. Alternatives to participating in this study include other medications or no treatment prior to surgery. Your study doctor will be able to explain these and other alternatives that are currently available to you to treat your fibroids. In addition, your study doctor will advise you whether or not you will need additional treatment.

The study doctor will tell you more about the risks and benefits of participating in this study as compared to the risks and benefits of alternative treatments. You are strongly encouraged to ask the study doctor or a member of the study staff if you have questions about the results of your laboratory tests and other diagnostic procedures.

What are the side effects of any treatment received when taking part?

The most commonly reported side effects in studies where pre-menopausal subjects were given several doses of asoprisnil for up to 6 months, were: headache, upper respiratory infection, nausea, vomiting, abdominal pain, breast tenderness, and muscle and back pain.

Ovarian cysts (abnormal sacs filled with liquid) were observed more frequently in subjects receiving asoprisnil compared with placebo. In most subjects, the ovarian cysts resolved or decreased in size while the subjects were still on asoprisnil and the subjects did not experience any physical symptoms. However, one subject that received asoprisnil and had a medical history of symptomatic ovarian cysts, did experience physical symptoms and was diagnosed with a hemorrhagic (bleeding) ovarian cyst. The cyst was surgically removed without complications.

One subject participating in a uterine fibroids clinical study developed a prolapsed fibroid (a fibroid that has moved from the uterus into the vagina). This condition may cause a sudden increase in vaginal bleeding over that which you may be experiencing. The subject underwent an uncomplicated removal of the fibroid with complete resolution of her bleeding. The study treatment group (placebo or asoprisnil) was not revealed and the subject remains in the study.

One subject in a uterine fibroids clinical study was found to have cancer in her uterus after receiving asoprisnil for approximately 9 months. MRI pictures of her uterus

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taken prior to starting treatment with asoprisnil appear to show the growth in which cancer was found after surgery. However, it is not known whether the cancer was present prior to the subject taking asoprisnil. The subject has undergone a hysterectomy without complications, and examination of surrounding fluid and tissue indicate that the cancer cells have not spread. Other notable events reported in subjects treated with asoprisnil include 1 case each of pancreatitis and pulmonary embolism (post-hysterectomy) that have resolved, and 1 case of seizure disorder.

Some patients experienced temporary alterations in hormone levels. This included a small to moderate increase in testosterone concentrations (male sex hormone) that was not associated with the development of masculine characteristics such as oily skin, acne or facial hair. While receiving asoprisnil, the normal cyclic changes within the endometrium (lining of the uterus) seen during the menstrual cycle may be suppressed.

There may be mild discomfort or pain associated with the gynaecologic examinations and TVU. The endometrial biopsy may cause pelvic discomfort during the procedure and for a few minutes afterwards, and/or uterine bleeding. Rarely, the procedure may result in perforation (tearing) of the uterus or infection, which could require hospitalisation for treatment. If a sedative is used during the biopsy, the study doctor will describe its risks to you but usually these include dizziness and feeling very tired afterwards.

Some women experience discomfort from the pressure applied when the speculum is inserted into the vagina or when the cervix is scraped to take a Pap smear. There may be temporary spotting or bleeding afterwards. You may experience discomfort from the pressure applied during a manual pelvic examination.

During the collection of blood samples, you may experience some pain and/or bruising on your arm where the blood is taken. In rare instances, a localised blood clot may form or an infection may occur. Some patients experience faintness during or shortly after having blood drawn.

Since the study medication is investigational, there may be other risks that are currently unknown or unforeseen. There is also the possibility of an allergic reaction to the study medication.

If you suffer from any of these or any other symptoms you should report them next time you meet the research doctor. If you have any worries at any time during the study you should contact your research doctor.

This information is provided, not to alarm you, but to help you in deciding whether or not to participate in the study. Your research doctor is aware of the possible risks and has determined that the potential benefits outweigh the possible risks. If you decide to take part, you will be insured for any study-induced injury.

What are the possible disadvantages and risks of taking part?

Women of Childbearing Potential:

Even though you may stop having menstrual periods during the study or you may have a delay in the start of your menstrual periods after stopping the study medication, you could still become pregnant if you do not use birth control. To date,

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several pregnancies have been reported during treatment or follow-up periods in short and long term asoprisnil studies.

The risk of asoprisnil to children born to mothers exposed during pregnancy to the medication is unknown at this time. With respect to the unborn baby, exposure during pregnancy may affect fetal survival. Furthermore, the possibility of malformations in the surviving fetus cannot be excluded. In laboratory animal studies, severe birth defects were seen in fetuses exposed to asoprisnil through the mothers.

As a result, women should not participate in this study if they are pregnant, breast-feeding, or actively trying to become pregnant. You will be required to have one or more pregnancy tests during the study and unless you have been sterilised or your partner has had a vasectomy, you must agree to use a medically proven double-barrier method of birth control (condoms with spermicide, sponge with spermicide, or diaphragm with spermicide) until the day of your hysterectomy. It is possible that you may not have menstrual periods during the time you are taking study medication or that your menstrual periods may be lighter, but even if this is the case, you must still use contraception until the date of your hysterectomy.

What are the possible benefits of taking part?

You may receive no or few benefits from this study. However, your symptoms of fibroids may be relieved by asoprisnil.

Study procedures such as physical examinations, electrocardiograms, transvaginal ultrasounds, biopsies, and laboratory tests are performed at no cost to you. Study medication is provided at no cost as well. We hope that the treatment will help you. However, this cannot be guaranteed. Information gained from this study may help develop new treatments for patients in the future.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the medication being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw, your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will asked to sign an updated consent form.

What happens when the research study stops?

The treatment is only available to you whilst on the study. Occasionally, the company making the medications will stop the research. If this happens you may have to withdraw from the study, but the research doctor will continue to care for you.

What if something goes wrong?

If you have any complaints during the study as to your treatment by the members of staff e.g. doctors, nurses etc you can report this through the NHS complaints procedure. The hospital can advise on this.

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Compensation for any injury caused by taking part in this study will be in accordance with the guidelines of the Association of the British Pharmaceutical Industry (ABPI). Broadly speaking the ABPI guidelines recommend that 'the sponsor', without legal commitment, should compensate you without you having to prove that it is at fault. This applies in cases where it is likely that such injury results from giving any new medication or any other procedure carried out in accordance with the protocol for the study. 'The sponsor' will not compensate you where such injury results from any procedure carried out which is not in accordance with the protocol for the study. Your right at law to claim compensation for injury where you can prove negligence is not affected.

Should you require further information, contact your research doctor (phone number and address can be found on page 11 of this document).

Will my taking part in this study be kept confidential?

If you consent to take part in the research, any of your medical records may be inspected by the company sponsoring and/or the company organising the research (Harrison Clinical Research) for purposes of analysing the results. People from regulatory authorities and the ethics committee may also look at your medical records to check that the study is being carried out correctly. Your name and address, however, will not be disclosed outside the hospital.

With your permission, your GP will be notified, by letter, of your participation in the study. In addition, other medical practitioners not involved in the study may also need to be notified. We will ask for your permission that these parties can be informed.

Data will be collected by the research doctor or a member of the research team in accordance with the Data Protection Act 1998. Data will be analysed and controlled by TAP Pharmaceutical Products Inc., and various government health agencies, in the USA and UK, which approve the use of new medications. The data collected from you for this study will be stored by the research doctor and TAP Pharmaceutical Products Inc. sponsoring the study. The data collected from you for this study will be stored on paper and electronically. The data will not be destroyed before the end of the study.

Your information may be passed onto researchers or regulatory authorities in countries that do not provide the same protection as the UK.

What will happen to the results of the research study?

When the study has been completed, a report will be prepared showing the results of the study. A copy of the report will be provided to the Ethics Committee.

Information contained in the report will be coded to protect identity. Your name and all other personal data, as well as that from other patients in this study will be kept confidential and, to the extent permitted by the applicable laws and/or regulations, will not be made publicly available. If the results of the study are published, your identity will always remain confidential.

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Who is organising and funding the research?

A US pharmaceutical company called TAP Pharmaceutical Products Inc. is funding this research study. This company is referred to as the 'sponsor'.

The Hospital NHS Trust is being paid to identify you as a suitable patient for participation in this study. This includes payments to cover the necessary expenses and laboratory tests and the salaries of the research staff involved.

You will not be paid for taking part in the study. However, you will be able to claim for travel expenses (see your research nurse or doctor for more details).

Who has reviewed the study?

To ensure the study is being conducted safely and ethically, the Local Research Ethics Committee has reviewed the study. This committee is a panel comprising medically qualified people and laypersons from the local community and is independent from the study doctor and the sponsor company (the company paying for the research to be done).

Contact for Further Information

Should you require it, further information can be obtained from Professor Critchley or other members of the research team at:

Tel: 0131 242 6441

Fax: 0131 242 6440

Address: Obstetrics and Gynaecology
Reproductive and Development Sciences
Centre for Reproductive Biology
University of Edinburgh
49 Little France Crescent
Old Dalkeith Road
Edinburgh EH16 4SB

We thank you for taking the time to read this information sheet, and (if you decide to do so) for taking part in this study.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from:

CERES
PO Box 1365
London N16 0BW

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Centre Number: 01 (Edinburgh)

Study Number: C02-003

Ethics Submission Number: 386/02/w

Patient Identification Number for this study: _____
(Screening Number)

Patient Initials: _____

CONSENT FORM

Title of Project: Study C02-003: A Phase II, 12-Week, Randomised, Double-Blind, Placebo Controlled Study to Evaluate the Effects of J867 on Uterine Artery Blood Flow and the Morphology of the Endometrium, Myometrium and Uterine Leiomyomata in Patients with Uterine Leiomyomata Scheduled for Hysterectomy.

Short Title of Project: J867 in women with uterine fibroids.

Name of Researcher:

(Please initial box)

1. I confirm that I have read and understand the information sheet dated 21 June 2004 (Version 4.3) for the above study and have had the opportunity to ask questions. I understand that I will receive a copy of this information sheet and a signed and dated consent form for my records. ☐

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2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

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3. I understand that sections of any of my medical notes may be looked at by responsible individuals from TAP Pharmaceutical Products Inc. (and/or their representatives) or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree that my GP and other medically qualified people who assist in my care can be notified of my participation in the trial. ☐

5. I agree that data about me relating to this study may be sent to countries that do not have data protection laws that are similar to those in the UK. I understand that my data will be made anonymous before the information leaves the hospital or research centre. ☐

6. I give permission for the sample of tissue collected to be stored for possible use in future studies. The precise nature of these studies will depend upon future scientific advances. I understand that some of these projects may be carried out by researchers other than the study researcher at the hospital, or members of the study team. ☐

7. I understand that if I withdraw from the study while on medication I will give my consent to collection of an endometrial biopsy as an outpatient. ☐

8. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

APPENDIX II

ETHICAL APPROVAL

Lothian NHS Board

Lothian Research Ethics Committee
Deaconess House
148 Pleasance
Edinburgh
EH8 9RS
Telephone 0131 536 9000
Fax 0131 536 9346
www.nhslothian.scot.nhs.uk

NHS Lothian

Prof Hilary Critchley
Reproductive Medicine
Room SU 304 NEW ROYAL INF
Univ of Edinburgh Med Sch
49 Little France Cres EDINBURGH

Date 06 November 2002
Your Ref
Our Ref LREC/2002/6/17

Enquiries to Joyce Clearie
Extension 89022
Direct Line 0131 536 9022
Email @lhb.scot.nhs.uk
US IRB No.: IRB00001462

Dear Prof Critchley,

A PHASE II 12-WEEK DOUBLE-BLIND PLACEBO-CONTROLLED STUDY TO EVALUATED THE EFFECTS OF J867 ON UTERINE ARTERY BLOOD FLOW AND THE MORPHOLOGY OF THE ENDOMETRIUM MYOMETRIUM AND UTERINE LEIOMYOMATA IN SUBJECTS WITH UTERINE LEIOMYOMATA SCHEDULED FOR HYSTERECTOMY.

Thank you for submitting the above research proposal for ethical review. The Paediatrics/Reproductive Medicine Research Ethics Committee of the Lothian Research Ethics Committee has reviewed this proposed research and has given it a favourable ethical opinion. Please note however that the committee felt that the patient information sheet should be clearer with regard to dosages i.e. page 1, **What is the purpose of the study?** section, paragraph 2 and page 6, **What is the drug or procedure that is being tested?** section, paragraph 3. An official Certificate of Ethical Opinion outlining the conditions of this opinion is enclosed together with a list of members present at the meeting. Please note that the LREC reference number LREC/2002/6/17 **must** be quoted on all correspondence. Correspondence received without the LREC reference number will be returned.

Under the terms of the Scottish Executive Health Department Research Governance Framework for Health and Community Care this opinion has been notified to the Research & Development Office of the relevant NHS Trust(s) where the research is intended to take place. It is the NHS Trust(s) from whom you must obtain management approval before any work on the proposed research can proceed.

07 NOV 2002

Details of the Lothian Research Ethics Committee and its documentation can be found on http://www.nhslothian.scot.nhs.uk/nhs_lothian/about_lothian_health/lrec/index.html

Yours sincerely

JOYCE CLEARIE
Committee Administrator

LOTHIAN RESEARCH ETHICS COMMITTEE

CERTIFICATE OF ETHICAL OPINION

LREC Reference Number: LREC/2002/6/17
Title: A Phase II 12-Week Double-blind Placebo-Controlled Study to Evaluated the Effects of J867 on Uterine Artery Blood Flow and the Morphology of the Endometrium Myometrium and Uterine Leiomyomata in Subjects with Uterine Leiomyomata Scheduled for Hysterectomy.
Researcher: Prof Hilary Critchley

The Paediatrics/Reproductive Medicine Research Ethics Committee of the Lothian Research Ethics Committee (the Committee) reviewed this proposed research and is of the opinion that it is ethical and appropriate to be carried out in the Lothian Area. This opinion encompasses all aspects of the application including the Patient/Subject Information Sheet and all other accompanying documentation provided.

The LREC application form, protocol, subject information sheet, information on compensation arrangements, payments to researchers and the provision of expenses to subjects (where appropriate) were reviewed and approved and the members of the Committee present at the meeting are shown on the attached *Membership List*.

This opinion is issued subject to the following conditions and is invalid if they are not followed:

- You must obtain appropriate management approval from the relevant NHS Trust(s) before starting the proposed research. It is the NHS Trust(s) that ultimately decide whether or not this research should go ahead taking account of the advice of the Local Research Ethics Committee.
- You must notify the Sub-Committee and the relevant NHS Trust(s), in advance, of any significant proposed deviation from the original protocol or application form and obtain approval for any such amendments using the *Amendment Approval Request Form.*
- You must submit reports to the Sub-Committee and the NHS Trust(s) once the study is underway if there are any unusual or unexpected results which raise questions about the safety of the research.
- You must report annually on successes, or difficulties, in recruiting subjects in order to provide useful feedback on perceptions of the study among patients and volunteers using the *Progress Report Form.*
- Where the study is terminated prematurely you must report within fifteen days indicating the reasons for early termination.
- You must submit a final report within three months of the completion of the study using the *Progress Report Form.*

Peter Reith
Secretary
Lothian Research Ethics Committee

Joyce Clearie
Administrator
Paediatrics/Reproductive Medicine
Research Ethics Committee

06 November 2002

APPENDIX III

SUPPLIERS OF GENERAL MATERIALS

TISSUE COLLECTION & HISTOLOGY	SUPPLIER
Liquid based cytology	ThinPrep® Pap Test, Cytyc Corp
Pipelle suction curette	Unimar Pipelle® Endometrial Suction Curette, Medscand
4% neutral buffered formalin (NBF)	See appendix II
Tissue-tek TEC Embedding Station	Sakura Finetek USA, Inc.
Thermo Shandon Gill 1 Hematoxylin	Anatomical Pathology International, Runcorn, Cheshire, UK
EA-50 (Eosin)	Cellpath Ltd, Newtown, Powys, UK
Olympus BX51 microscope	Olympus UK Ltd, Southend-on-Sea, Essex, UK

IMMUNOHISTOCHEMISTRY	SUPPLIER
Histoclear	National Diagnostics, Atlanta, Georgia, USA
Phosphate buffered saline (PBS)	Sigma tablets (see appendix II)
PBS + Tween 20 (PBST)	Sigma tablets (see appendix II)
Sodium citrate	See appendix II
Pressure cooker	Model: CLIPSO, Tefal, Nottingham, UK
Bond-X Immunohistochemistry Staining System	Vision BioSystems, Newcastle Upon Tyne, UK
Bond detection system reagents: - 3% hydrogen peroxide - post primary polymer penetration enhancer - poly-horse radish peroxidase (HRP) anti-mouse/rabbit IgG - 3,3'-diaminobenzidine tetrahydrochloride (DAB) - hematoxylin	Vision BioSystems, Newcastle Upon Tyne, UK
Hydrogen peroxide solution (30%)	BDH Laboratory supplies, Poole, UK
Non-immune horse serum	Vector laboratories, Peterborough, UK
Biotinylated horse anti-mouse antibody	Vector laboratories, Peterborough, UK
Avidin-biotin-peroxidase complex (ABC-HRP)	Vector laboratories, Peterborough, UK

Avidin/Biotin blocking kit	Vector laboratories, Peterborough, UK
Non-immune goat serum	Autogen Bioclear
Bovine serum albumin	Sigma, Poole, Dorset, UK
Anti-rabbit envision kit (EnVision+ System-HRP)	DAKO Cytomation, Cambridge, UK
3, 3'-diaminobenzidine (DAB)	DAKO, Cambridge, UK
Harris's haematoxylin	Pioneer Research Chemicals Ltd, Colchester, UK
Xylene	BDH Laboratory supplies, Poole, UK
Pertex	Cellpath plc, Hemel Hempstead, UK
Axioskop 2 / Cell Imaging System	Carl Zeiss Ltd
Stereology software (Image-Pro plus 4.5.1 with Stereology-Pro 5.0)	Media Cybernetics UK, Wokingham, Berkshire, UK
Olympus BH-2 microscope	Prior Scientific Instruments Ltd, Cambridge, UK

PRIMARY ANTIBODIES	**SUPPLIER**
Mouse anti-Ki-67 / NCL-Ki67-MM1	Novocastra, Newcastle-upon-Tyne, UK
Rabbit Anti-phospho-histone H3	Upstate Biotechnology, Buckingham, UK
Mouse anti-PTEN (NCL-PTEN)	Novocastra, Newcastle-upon-Tyne, UK
Mouse anti-CD56	Zymed Laboratories, Cambridge, UK
Mouse anti-ERα: Clone 1D5	DAKO, Cambridge, UK
Mouse anti-ERβ1	Serotec, Oxford, UK
Mouse anti-PR: specific to both PR isoforms	Novocastra, Newcastle upon Tyne, UK
Mouse anti-GCR	Novocastra, Newcastle upon Tyne, UK
Rabbit anti-AR (F-39)	Novocastra, Newcastle upon Tyne, UK
Non-immune mouse IgG	Vector Laboratories, Peterborough, UK
Non-immune mouse IgG1	Sigma, Poole, Dorset, UK
Non-immune mouse IgG1 kappa	Sigma, Poole, Dorset, UK
Non-immune rabbit IgG	Vector Laboratories, Peterborough, UK
Antibody diluent	Vision BioSystems, Newcastle Upon Tyne, UK

RNA EXTRACTION	SUPPLIER
RNAlater® Solution (RNA stabilization)	Ambion Inc, Austin, Texas, USA
Trizol RNA isolation reagent	Invitrogen, Paisley, UK
Hand held homogeniser (Polytron PT 1200B)	Kinematica, Switzerland
Phase lock gel tubes	Eppendorf, Hamburg, Germany
Chloroform	Sigma, Poole, Dorset, UK
Isopropanol	Sigma, Poole, Dorset, UK
Ethanol (molecular biology grade)	BDH Laboratory supplies, Poole, UK
RNA storage solution	Ambion Inc, Austin, Texas, USA
Qiagen RNeasy mini kit columns	Qiagen Sciences, Maryland, USA

REAL TIME Q-RT-PCR	SUPPLIER
DNAse 1, Amplification grade kit (includes DNAse buffer and EDTA)	Invitrogen Life technologies, Paisley, UK
Agilent RNA 6000 Nano Kit	Agilent Technologies, Waldbronn, Germany
Taqman reverse transcription reagents	Applied Biosystems, Cheshire, UK
Taqman universal PCR mastermix	Applied Biosystems, Cheshire, UK
Ribosomal RNA control reagents	Applied Biosystems, Cheshire, UK
Primer/probe sets for sequences of interest	Applied Biosystems, Cheshire, UK or Biosource International Ltd, UK
Primer/probe set for IL-15	Universal Probe Library, Roche Diagnostics, Germany
ABI Prism 7900	Applied Biosystems, Cheshire, UK

APPENDIX IV

RECIPES

4% NBF		
Weight / Volume	**Chemical name**	**Supplier**
6.5 g	Na_2HPO_4	BDH
4.5 g	$Na_2PO_4.2H_2O$	BDH
100 ml	40% formaldehyde	BDH
900 ml	Distilled water	

0.01M PBS pH 7.4		
Weight / Volume	**Chemical name**	**Supplier**
5 tablets	PBS	Sigma
1 l	Distilled water	

0.1M Sodium citrate		
Weight / Volume	**Chemical name**	**Supplier**
29.41 g	Tri-sodium citrate	BDH
0.1 g	Sodium azide	Sigma
700 ml	Distilled water	
pH to 6 and make up to 1 l with distilled water		
Dilute 1:10 for working 0.01M solution		

0.01M PBST pH 7.4		
Weight / Volume	**Chemical name**	**Supplier**
5 tablets	PBS	Sigma
1 l	Distilled water	
8 g	NaCl	BDH
100 µl	Tween 20	Sigma

APPENDIX V

3 HOUR POLYMER DEFINE PROTOCOL

ID: 10003

Created by: VISION-5T27W1UO\BondService

Supplier of all reagents: Vision BioSystems

Temperature throughout staining process: Ambient

Step 1	Reagent - Peroxide Block	5 minutes
Step 2	Bond Wash solution	
Step 3	Bond Wash solution	
Step 4	Bond Wash solution	
Step 5	Reagent - Primary antibody	180 minutes
Step 6	Bond Wash solution	
Step 7	Bond Wash solution	
Step 8	Bond Wash solution	
Step 9	Reagent - Post primary	8 minutes
Step 10	Bond Wash solution	2 minutes
Step 11	Bond Wash solution	2 minutes
Step 12	Bond Wash solution	2 minutes
Step 13	Reagent – Polymer	8 minutes
Step 14	Bond Wash solution	2 minutes
Step 15	Bond Wash solution	2 minutes
Step 16	Deionized water	
Step 17	Reagent – Mixed DAB Define	
Step 18	Reagent – Mixed DAB Define	10 minutes
Step 19	Deionized water	
Step 20	Deionized water	
Step 21	Deionized water	
Step 22	Reagent – Hematoxylin	5 minutes
Step 23	Deionized water	
Step 24	Bond wash solution	
Step 25	Deionized water	

APPENDIX VI

3 HOUR POLYMER REFINE PROTOCOL

ID: 10004
Created by: BondPowerUser

Supplier of all reagents: Vision BioSystems
Temperature throughout staining process: Ambient

Step 1	Reagent - Peroxide Block	5 minutes
Step 2	Bond Wash solution	
Step 3	Bond Wash solution	
Step 4	Bond Wash solution	
Step 5	Reagent - Primary antibody	180 minutes
Step 6	Bond Wash solution	
Step 7	Bond Wash solution	
Step 8	Bond Wash solution	
Step 9	Reagent - Post primary	8 minutes
Step 10	Bond Wash solution	2 minutes
Step 11	Bond Wash solution	2 minutes
Step 12	Bond Wash solution	2 minutes
Step 13	Reagent – Polymer	8 minutes
Step 14	Bond Wash solution	2 minutes
Step 15	Bond Wash solution	2 minutes
Step 16	Deionized water	
Step 17	Reagent – Mixed DAB Refine	
Step 18	Reagent – Mixed DAB Refine	10 minutes
Step 19	Deionized water	
Step 20	Deionized water	
Step 21	Deionized water	
Step 22	Reagent – Hematoxylin	5 minutes
Step 23	Deionized water	
Step 24	Bond wash solution	
Step 25	Deionized water	

APPENDIX VII

PUBLICATIONS

- Published by **Faculty of Sexual and Reproductive Healthcare**
(Reproduced with permission)

Progesterone receptor modulators in gynaecological practice

Wilkens J, Critchley H
Journal of Family Planning and Reproductive Health Care
Volume 36, No 2, pp 87-92, 2010

- Published by **Oxford University Press**
on behalf of the European Society of Human Reproduction and Embryology

Effect of asoprisnil on uterine proliferation markers and endometrial expression of the tumour suppressor gene, PTEN

Wilkens J, Williams ARW, Chwalisz K, Han C, Cameron IT, Critchley HOD
Human Reproduction, Volume 1, No 1, pp 1-9, 2009

- Published by **The Endocrine Society**

Effects of the selective progesterone receptor modulator asoprisnil on uterine artery blood flow, ovarian activity and clinical symptoms in patients with uterine leiomyomata scheduled for hysterectomy

Wilkens J, Chwalisz K, Han C, Walker J, Cameron IT, Ingamells S, Lawrence AC, Lumsden MA, Hapangama D, Williams ARW, Critchley HOD
The Journal of Clinical Endocrinology & Metabolism
Volume 93, No 12, pp 4664-4671, 2008

- Published by **Oxford University Press**
  on behalf of the European Society of Human Reproduction and Embryology

  The effects of the selective progesterone receptor modulator asoprisnil on the morphology of uterine tissues after 3 months treatment in patients with symptomatic uterine leiomyomata

  Williams ARW, Critchley HOD, Osei J*, Ingamells S, Cameron IT, Han C, Chwalisz K
  Human Reproduction, Volume 22, No 6, pp 1696-1704, 2007

- Published by **Wolters Kluwer Health / Lippincott Williams & Wilkins**
  (Reproduced with permission)

  Menorrhagia, mechanism and targeted therapies

  Osei J*, Critchley H
  Current Opinion in Obstetrics & Gynecology
  Volume 17, No 4, pp 411-418, 2005

(* Osei = Previous surname of author)

Progesterone receptor modulators in gynaecological practice

Julia Wilkens, Hilary Critchley

Introduction

The sex steroid progesterone plays a major role in the regulation of female reproductive activity. Circulating levels of progesterone are maximal in the secretory phase of the menstrual cycle, its main source being the corpus luteum. The effects of progesterone are mediated via the progesterone receptor (PR), which is essential not only for the co-ordination of ovulation, implantation and the maintenance of pregnancy but also for breast development and sexual behaviour. Within the uterus, progesterone predominantly contributes to the regulation of endometrial function. Progesterone is crucial in preparation for implantation by decidualisation, during pregnancy and during the process of menstruation, which occurs when progesterone levels fall in the absence of a conceptus. Since the discovery of natural progesterone in the mid-1930s,[1] many synthetic progestogens have been developed and introduced into clinical practice. As contraceptive agents, these compounds have revolutionised fertility control. Progestogens also have an established role in the management of benign gynaecological conditions such as menstrual disturbances and endometriosis, as well as in hormone replacement therapy and assisted reproductive technology.

The discovery of the progesterone receptor[2,3] enabled the development of synthetic compounds binding to the PR with agonistic but also with antagonistic properties. The first of these to be described in 1981 was mifepristone (RU 486), a PR and glucocorticoid receptor (GR) antagonist.[4] Reports soon followed on its effect on menstruation,[5] highlighting its potential for cycle regulation and birth control.[6] It was established that mifepristone could induce menstruation by interrupting the luteal phase of the menstrual cycle. Its potency to disrupt early pregnancy was then recognised, leading to its clinical application in the termination of pregnancy.[7] Various related compounds exerting their effect via the PR have subsequently been developed and are reviewed here.

Development of PRMs

The initial aim in developing progesterone receptor modulators (PRMs) was to find compounds with more profound P antagonistic potency but less antiglucocorticoid activity than mifepristone. The applications envisaged in the early stages were fertility control and the treatment of breast cancer.[8,9] The major therapeutic potential of PRMs for the management of benign gynaecological conditions became apparent when their endometrial antiproliferative effect inducing endometrial atrophy and amenorrhoea was demonstrated, initially in non-human primates and rabbits.[10,11] Unfortunately, the significant abortifacient activity of progesterone receptor antagonists (PAs) led to the delay and obstruction of development of mifepristone and similar compounds with progesterone antagonistic properties for other clinical applications. Large pharmaceutical companies distanced themselves from the perceived negative image of a drug that was capable of terminating a pregnancy. Further introduction of PRMs into clinical practice was also precluded by concerns regarding safety of the endometrium, as it may potentially be exposed to the effects of unopposed estrogens, and concerns regarding their antiglucocorticoid activity at higher doses.

A drug discovery programme was initiated to develop a compound with partial PR agonistic and antagonistic properties on the assumption that this would eliminate the concerns regarding PRMs.[12] A number of compounds with varying degrees of PR agonist and antagonist properties were synthesised (Figure 1) and those with partial and mixed agonist/antagonist activity were classified as selective progesterone receptor modulators (SPRMs). These demonstrated high PR binding affinity and PR specificity with much reduced antiglucocorticoid activity compared to mifepristone. Their more favourable effect on the endometrium was attributed to their partial PR agonistic activity.[13,14] The SPRM, asoprisnil (J867), was selected for further clinical development as it demonstrated particularly pronounced PR agonistic properties and absence of labour-inducing or abortifacient activity.[12]

Many potential indications for PRMs in benign gynaecology have been proposed including the management of menstrual disturbances, uterine fibroids and endometriosis.[15,16] Most clinical experience to date has been with asoprisnil and mifepristone. Randomised double-blind placebo-controlled trials have also been conducted with ulipristal (CDB-2914) and proellex (CDB-4124).[17] For many years, mifepristone was the only compound licensed for clinical use. Recently, ulipristal has received a licence for emergency contraception.[18,19]

A number of other PRMs have been developed including onapristone, Org 31710, Org 33628, ZK 137 316, ZK 230 211 among others. Preclinical[20–23] and clinical[24] studies have been published with findings of dose-dependent suppression of menstrual and ovarian cyclicity.[21,23] Profound reduction of menstrual blood loss has been demonstrated consistently. Studies in macaques have been conducted with ZK 230 211 administered via an intrauterine system (IUS) with similar results.[22] The use of ZK230 211 administered via an IUS in humans with a view to its potential in contraception and in the management of benign gynaecological conditions has been evaluated in one pilot study.[24] This study demonstrated that intrauterine delivery of ZK 230 211 is feasible, as significant endometrial levels of the drug were detected.

This review focuses on the evidence for the use of the PRMs that have been administered in clinical trials to date.

Endometrial effects of PRMs

The discovery of the endometrial antiproliferative effect of PRMs was an important milestone in their development.[11,25] It was also unexpected, as compounds

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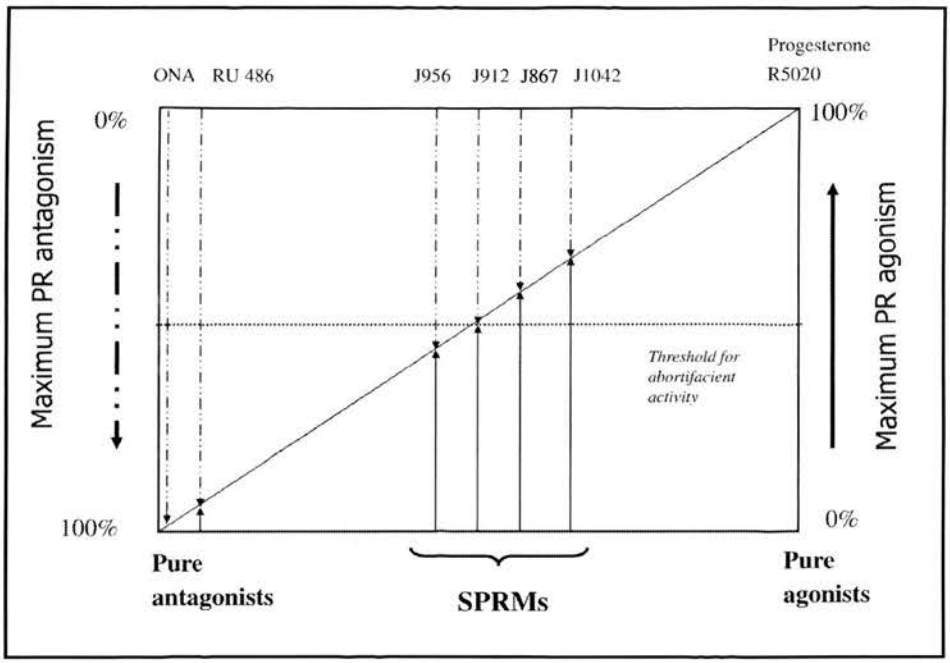

Figure 1 Examples of selective progesterone receptor modulators (SPRMs) exhibiting varying degrees of progesterone receptor (PR) agonist and antagonist properties, including those with partial and mixed agonist/antagonist activity. ONA, onapristone; RU486, mifepristone; J867, asoprisnil. Figure reproduced, with permission, from Schubert G, Elger W, Kaufmann G, Schneider B, Reddersen G, Chwalisz K, Discovery, chemistry, and reproductive pharmacology of asoprisnil and related 11beta-benzaldoxime substituted selective progesterone receptor modulators (SPRMs). *Semin Reprod Med* 2005; **23**: 58–73. © Thieme Medical Publishers

with PR antagonistic activity had been anticipated to result in unopposed estrogenic effects on the endometrium. A number of studies had described a significant proportion of women developing endometrial hyperplasia when mifepristone was used for the management of uterine fibroids at doses of 5–50 mg daily for 3–6 months.[26–28] A report was also published of significant endometrial hyperplasia following 6 months courses of mifepristone at 400 mg per day.[29] On further histological examination, however, in most cases the changes described as hyperplastic consist of cystically dilated glands,[30] which contrary to the glands in endometrial hyperplasia, do not exhibit increased mitotic indices. There have been no reports of cytological atypia. Administration of mifepristone in low doses (2–5 mg) for 120 days has been reported to reduce endometrial proliferation markers.[31] Asoprisnil has not been found to cause endometrial hyperplasia after administration for up to 12 weeks. A study into the effects of 10 or 25 mg asoprisnil given for 12 weeks on endometrial proliferation markers demonstrated that asoprisnil does not induce proliferation, and indeed the stromal expression of the proliferation marker Ki-67 was significantly decreased. In addition, the expression of the tumour suppressor gene PTEN, previously described as a gatekeeper of endometrial carcinogenesis,[32] was unaffected and specifically not suppressed.[33]

It has now been recognised that the endometrial morphological features described with PRMs are a unique class effect and are not consistent with the previously recognised histological changes through the normal cycle.[34,35] New diagnostic criteria to describe the features observed with PRM administration have been developed with the intention of raising the awareness of pathologists when assessing endometrial morphology after exposure to this class of compound.[36] To date, no pre-malignant lesions have been described.

Potential clinical applications for PRMs

Heavy menstrual bleeding

Menstrual disturbances have an impact on the quality of life of many women. The prevalence of menstrual complaints has increased over the last century with earlier menarche, increased life expectancy, advances in fertility control and shorter episodes of lactational amenorrhoea. With the current trend for women to postpone plans for a family to their fourth or even fifth decade, surgical intervention is often not an option. Hence, there is an increasing requirement for an acceptable form of medical management.[37,38] Suppression of menstruation may be desirable not only for women suffering from excessive menstrual blood loss but also as a personal option for any woman between menarche and menopause. The perceived benefits of amenorrhoea include the abolition of heavy blood loss and painful periods and a reduced risk of anaemia. A questionnaire survey of 1001 women attending a family planning clinic showed that the reduction of periods associated with certain forms of contraception was highly acceptable.[39] It has even been argued that menstruation should be optional for all women.[40–43] PRMs may offer this option once it has been established that long-term administration is safe.

The medical management options for heavy menstrual bleeding (HMB) have been reviewed previously.[44] Currently, most involve the administration of progestogens, either orally, parenterally or via an IUS. The side effect most commonly responsible for discontinuation of such therapy is unscheduled bleeding. Most studies assessing the effect of PRMs on bleeding pattern have been carried out in patients with benign uterine pathology such as uterine fibroids, which are known to contribute to the symptom of HMB. However, the effect of asoprisnil was also evaluated in 60 women with regular menstrual cycles and no uterine pathology in a Phase I double-blind dose-escalation study. They were administered doses of asoprisnil varying from 5 mg once daily to 50 mg twice daily for 28 days commencing during the first 4 days of their cycle. As a result, cycle lengths were increased, and the onset of menstruation was significantly delayed with doses at or above 10 mg once daily.[45] Suppression of menstruation with asoprisnil has been found to be reversible and not associated with the adverse systemic side effects of estrogen deprivation as seen with gonadotropin-releasing hormone (GnRH) analogues.[12,46] The prolongation of the menstrual cycle in this Phase I study occurred even in the presence of a normal luteal phase and luteolysis, indicating that the endometrium is a direct target.[45] Asoprisnil may have clinical advantages over continuous progestogen treatment, as reports of unscheduled bleeding with PRMs have been rare.

Uterine fibroids

Current options of medical management for symptoms associated with uterine fibroids are very limited due to unfavourable success rates and side effect profiles. HMB is the commonest symptom requiring intervention and is often refractory to administration of progestogens, particularly if the uterine cavity is enlarged and distorted. Any symptoms due to the mass of the fibroids such as pelvic pressure or discomfort are even less amenable to pharmacological intervention. GnRH analogues may only be administered on a temporary basis due to their hypo-estrogenic side effects. Uterine artery embolisation (UAE) is a potentially fertility-conserving and non-surgical management option and has recently been compared to hysterectomy with favourable results.[47] Longer-term follow-up studies of UAE, particularly regarding its effect on subsequent pregnancies, are awaited.

PRMs may prove to be an important advance in the medical management of symptomatic uterine fibroids as the clinical effects of this class of compounds are dramatic.[48,49] In addition to their beneficial effect on bleeding patterns, PRMs reduce the size of the fibroids and therefore have the potential to alleviate symptoms due to fibroid mass. Favourable results have been reported in studies that included assessments of the impact on patients' leiomyoma-specific quality of life.[50,51]

Mifepristone has been described to significantly improve bleeding patterns in women with uterine fibroids. Reports of amenorrhoea vary and appear to be dose dependent, but range from 60–100% with doses of 5–50 mg daily.[26–28,52] A recent study using a low dose of 5 mg mifepristone for 6 months found an amenorrhoea rate of 41%.[51] Other fibroid-related symptoms such as dysmenorrhoea and pelvic pressure have also been seen to respond favourably to the administration of mifepristone.[27] Reduction in the size of fibroids by 26–74% has been described with administration of 5–50 mg mifepristone once daily for 3–6 months.[27] The evidence regarding the dose dependency of this effect is currently inconclusive. Whilst no consistent correlation between dose and response could be found in one review,[27] a study specifically investigating the dose–response effect concluded that only doses of at least 25 mg daily achieved a clinically significant decrease in fibroid volume.[53] The reduction in the size of fibroids may also be related to the duration of exposure to mifepristone.[27] Recent randomised placebo-controlled trials in women with symptomatic fibroids have reproduced the findings of decreased fibroid volumes and a significant reduction in menstrual blood loss, resulting in increased haemoglobin levels after treatment with 5–50 mg mifepristone once daily for 3–6 months.[28,30,51]

Phase II studies with asoprisnil in women with uterine fibroids reaffirmed the observation that this compound induces reversible amenorrhoea. In a double-blind, placebo-controlled study, doses of 5, 10 or 25 mg of asoprisnil were administered orally to women with uterine fibroids for 12 weeks.[54] Duration and intensity of uterine bleeding were significantly reduced in a dose-dependent manner, and no episodes of unscheduled bleeding were reported. Amenorrhoea was achieved in over 83% of patients with the highest dose of 25 mg. In addition to the suppression of both normal and heavy menstrual bleeding, a reduction in the volume of the largest fibroid (by 36% after 12 weeks with 25 mg asoprisnil) was demonstrated, resulting in a dose-dependent improvement of pressure symptoms such as bloating and pelvic pressure.[48–50] In a subsequent and similar double-blind, placebo-controlled study with 10 or 25 mg of asoprisnil for 12 weeks, these findings were reproduced with a particularly impressive reduction in menstrual blood loss, a decrease in fibroid size and a significant improvement in quality of life.[50] In this study, the menstrual blood loss was semi-quantitatively assessed with a menstrual pictogram, which showed that between the pre-treatment cycle and the final month of treatment there was a difference in blood loss of –154 and –215 ml in the 10 and 25 mg asoprisnil groups, respectively. Considering the traditional definition of HMB as menstrual loss over 80 ml,[55] this was a dramatic improvement. Even in this cohort of women presenting with excessive menstrual bleeding, amenorrhoea was achieved in 91% of patients with a daily dose of 25 mg asoprisnil.

The effect of CDB-2914 administered for 3 months in doses of 10 and 20 mg to women with symptomatic fibroids has been evaluated in a randomised, placebo-controlled trial. The higher dose of 20 mg achieved amenorrhoea in all patients. As well as suppression of menstruation and ovulation, CDB-2914 was found to significantly reduce fibroid volumes by 21–36% and to improve quality of life, comparable to the results of studies with other PRMs.[56]

The mechanism of the effect of PRMs on fibroid size has not yet been fully elucidated. Reduced uterine artery blood flow in women with symptomatic uterine fibroids has been described with both mifepristone[57] and asoprisnil[50] and may contribute to the decrease in tumour size. Asoprisnil also appears to target uterine leiomyoma cells directly, resulting in restriction of proliferation and induction of apoptosis, whilst leaving normal myometrial cells unaffected.[58]

Endometriosis

As with other benign gynaecological conditions, medical management of endometriosis is currently largely dependent upon administration of progestogens. Estrogen deficiency restricts long-term use of GnRH analogues. Not only is satisfaction with medical management often limited by the side effects, but symptom control may also remain suboptimal.

The results of studies of PRMs in women with endometriosis are promising.[13,59] Mifepristone has been shown to have a significant beneficial effect on symptoms and extent of disease with administration of 50 mg daily for 6 months.[59] The rationale for the use of asoprisnil in the management of endometriosis is based on the presumed effects of tissue-selective inhibition of endometrial proliferation and suppression of endometrial bleeding by targeting the endometrial vasculature directly.[13,49] The finding of tissue-specific suppression of endometrial prostaglandin production in preclinical studies also appeared promising with regard to the potential of asoprisnil to ameliorate endometriosis-associated pain.[10,60] Phase II studies with asoprisnil have been conducted in women with pelvic pain due to endometriosis. In a randomised, placebo-controlled study, doses of 5, 10 or 25 mg asoprisnil were administered for 12 weeks to women with a laparoscopic diagnosis of endometriosis who suffered moderate or severe pain. All three doses significantly reduced non-menstrual pelvic pain and dysmenorrhoea compared to placebo.[49]

Contraception

Mifepristone strongly antagonises all effects of progesterone, including endometrial preparation for implantation and maintenance of pregnancy. Hence, not surprisingly, its first clinical application was for termination of pregnancy, which remains its only licensed indication.[61,62] Mifepristone has also been demonstrated to

act as a highly effective postcoital contraceptive agent.[63–65] The potential of ulipristal for use in emergency contraception was also explored, and it was found to be at least as effective as the progestogen, levonorgestrel (LNG).[66] Ulipristil has received a product licence for this indication and is therefore the second PRM to become available for clinical use.

The concept of administering mifepristone as an estrogen-free contraceptive pill has also been evaluated.[67,68] Clinical trials have been conducted administering 2 or 5 mg of mifepristone daily for 4–6 months, demonstrating it to be an effective oral contraceptive agent, inhibiting ovulation and inducing amenorrhoea in the majority of cases.[69] When compared to progestogen-only pills, the effect on the bleeding pattern was significantly more favourable, with more cases of amenorrhoea and a lower incidence of spotting.[70] There is a potential for PRMs to be developed for use in fertility control, if their advantages can be more widely appreciated and if they can be divorced from their image as abortifacients.

PRMs have been administered as a therapeutic intervention for the management of unscheduled bleeding with progestogen-only contraception (POC).[71–73] Breakthrough bleeding is a frequent reason for discontinuing POC.[74,75] In one study, the discontinuation rate of new users of the LNG-releasing intrauterine system (LNG-IUS) due to bleeding problems was found to be 16.7% over 5 years. As is well known, this side effect is most prevalent during the first months post-insertion and most patients who stop using the LNG-IUS due to unscheduled bleeding do so during the first year (10.5%).[76] PRMs have been proposed to ameliorate this effect.[77] Even a single dose of mifepristone (200 mg) has been shown to reduce bleeding episodes in users of a LNG-releasing subdermal contraceptive implant.[73] Org 31710 also appeared to regularise the bleeding pattern when administered monthly in addition to a desogestrel-only contraceptive pill.[78] However, suppression of unscheduled bleeding has not been consistently demonstrated, and the implications for contraceptive efficacy also remain to be clarified.[79,80] One study administering CDB-2914 to new LNG-IUS users in an attempt to prevent breakthrough bleeding found that the initial beneficial effect was temporary. With ongoing treatment, the placebo group appeared to have a more favourable bleeding pattern, even though there were no significant differences in acceptability measures.[79] This study highlighted the important difference between prevention and treatment of an undesired side effect and concluded that there is possibly more potential for PRMs to improve than to prevent unscheduled bleeding in users of POC.[79]

Other indications

Alternative applications of PRMs beyond the context of gynaecology have also been proposed, either utilising the antiglucocorticoid effects of some compounds, or in non-gynaecological conditions that are sensitive to sex steroids. The use of PAs in the treatment of breast cancer was envisaged very soon after their discovery.[81] Since then, many other applications for PRMs have been considered and reviewed.[82] Clinical trials have mostly been conducted with mifepristone.

Preclinical and clinical studies exploring the potential of mifepristone for the management of breast cancer succeeded in demonstrating a response of tumour growth inhibition.[83] A Phase II study with daily administration of 200 mg mifepristone in women with untreated metastatic breast carcinoma concluded that its use as a single agent could not be supported.[84] However, an additive antiproliferative effect was demonstrated when PAs were combined with anti-estrogens such as tamoxifen or with an aromatase inhibitor, suggesting that PRMs may have a place in the endocrine therapy of breast cancer as part of a combination regime.[85] The potential of PAs for the prevention of breast cancer has also been evaluated. In premenopausal women, the administration of 50 mg mifepristone every other day for 3 months has been shown to block breast epithelial cell proliferation, implying a possible protective effect.[86] In another study, mice lacking the rodent version of the breast cancer susceptibility gene BRCA1 were given mifepristone, which inhibited mammary tumorigenesis.[87] The results of these studies appear to support a potential preventive role for PRMs, particularly in women who have been identified as being at increased risk of developing breast cancer.

Mifepristone has also been used in cases of inoperable meningioma after it was established that this tumour type is modulated by female sex hormones and commonly expresses PR.[88,89] There may be further benefits in the management of other tumour types,[90] and a response of human ovarian carcinoma cell lines has been reported in an *in vitro* study.[91] Whilst the antiglucocorticoid activity of mifepristone may result in commonly undesired side effects, it also constitutes the basis for its advantages in the management of Cushing's syndrome.[92,93] Overall, the broad medical applicability of mifepristone has been appreciated for almost two decades, but political issues surrounding its association with termination of pregnancy have hampered further development.[94]

Summary

Preclinical and clinical studies have demonstrated the potential role for PRMs in the management of benign gynaecological conditions. Mifepristone is licensed for use in termination of pregnancy but its potential advantages for use in contraception, management of HMB, uterine fibroids and endometriosis as well as in non-gynaecological and oncological conditions have also been highlighted. Further development of related compounds has been obstructed by the controversial political issues regarding their abortifacient properties. The potential of asoprisnil to become a novel treatment for HMB, symptomatic uterine fibroids and endometriosis has been demonstrated and accompanied by a favourable safety and tolerability profile in all clinical studies to date. Ulipristal has recently been licensed for use in postcoital contraception, and the potential applicability of other PRMs has also been explored.

Early studies with mifepristone reported endometrial morphological changes as hyperplastic, but these have subsequently been described in further detail as cystic glandular dilatation without increased mitotic activity. No pre-malignant changes have been reported in response to treatment with PRMs. The unique morphological features demonstrated in endometrium following administration of PRMs are now recognised.[36] In the future, it is hoped that long-term studies will confirm the safety as well as the efficacy of these compounds.

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ORIGINAL ARTICLE *Gynaecology*

Effect of asoprisnil on uterine proliferation markers and endometrial expression of the tumour suppressor gene, PTEN

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BACKGROUND: The selective progesterone receptor modulator asoprisnil suppresses uterine bleeding and decreases leiomyoma volume while maintaining follicular phase estrogen concentrations. For safety of potential clinical applications, any proliferative effect of asoprisnil on uterine tissues, particularly endometrium, needs to be established.

METHODS: In a double-blind, randomized, placebo-controlled study (continuation of previously published trial No. NCT00150644 (Williams *et al.*, 2007 and Wilkens *et al.*, 2008)), 33 patients with symptomatic uterine leiomyomata received placebo, 10 or 25 mg asoprisnil daily for 12 weeks before hysterectomy. Proliferation markers Ki-67 and anti-phospho-histone H3 (PH3) were immunolocalized in endometrium, myometrium and leiomyoma tissue. Endometrial PTEN (phosphatase and tensin homologue, a tumour suppressor gene) expression was also assessed by immunohistochemistry. PH3-positive glandular and stromal cells were counted per measured endometrial area. Endometrial Ki-67 expression was assessed using stereological methods. Stained myometrial and leiomyoma cells were counted per 10 fields (×250). PTEN immunostaining was quantified using a histoscore. Each asoprisnil group was compared with placebo (secretory phase) with significance at 0.05 level.

RESULTS: Endometrial epithelial proliferation and PTEN expression were not significantly different between placebo and asoprisnil groups. Decreased stromal Ki-67 expression ($P < 0.05$) suggested any effect of asoprisnil on endometrial proliferation to be inhibitory. Immunolocalization of PTEN expression was not different between treatment groups in any tissue compartments. Myometrial Ki-67 expression decreased following asoprisnil 25 mg ($P < 0.05$).

CONCLUSIONS: Asoprisnil does not induce proliferation of uterine tissues and does not suppress endometrial PTEN expression.

Key words: asoprisnil / uterine tissues / proliferation / phosphatase and tensin homologue

Introduction

Selective progesterone receptor modulators (SPRM) are a novel class of progesterone receptor ligands exhibiting mixed and/or partial agonist and antagonist activity. Asoprisnil is the first SPRM that has been clinically evaluated in patients with symptomatic uterine fibroids and endometriosis (Chwalisz *et al.*, 2005b; Wilkens *et al.*, 2008). Asoprisnil is a 11β-benzaldoxime-substituted steroidal SPRM that shows a high degree of uterine selectivity (Schubert *et al.*, 2005).

Previously conducted clinical studies have shown asoprisnil to reversibly suppress uterine bleeding by primarily targeting the endometrium (Chwalisz *et al.*, 2005a). Asoprisnil has further been demonstrated to reduce the volume of uterine fibroids in a dose-dependent manner (Chwalisz *et al.*, 2005b). It has, therefore, potential for the medical management of symptomatic uterine fibroids, which commonly cause heavy menstrual bleeding as well as pressure-related symptoms.

Asoprisnil and other structurally related SPRMs demonstrated endometrial antiproliferative effects in non-human primates (Chwalisz *et al.*, 2005b; Schubert *et al.*, 2005). In cynomolgus monkeys, asoprisnil induced profound endometrial atrophy in the presence of early luteal phase estrogen concentrations (Brenner *et al.*, 2005). This effect was accompanied by a decrease in the proliferation markers Ki-67 and

anti-phospho-histone H3 (PH3). Similar endometrial antiproliferative effects were described in the macaque endometrium with mifepristone (Slayden and Brenner, 1994) and other progesterone receptor antagonists (antiprogestogens) including ZK 137 316 (Slayden *et al.*, 1998). The exact mechanism of the endometrial antiproliferative effects of SPRMs and antiprogestogens remains poorly understood in spite of extensive studies conducted in non-human primates (Brenner and Slayden, 2005).

We previously described the effects of asoprisnil on the morphology of the endometrium, myometrium and leiomyomata in patients with symptomatic uterine leiomyomata treated with asoprisnil for 3 months prior to hysterectomy (Williams *et al.*, 2007). The results of this study showed that asoprisnil suppressed endometrial proliferation, as evidenced by a low mitotic index in endometrial glands and stroma, which is consistent with the studies conducted in non-human primates. However, differences in endometrial morphology in response to asoprisnil treatment were noted in humans compared with macaques. Although this study also revealed a decrease in endometrial thickness, unique endometrial morphology was observed in women treated with asoprisnil for 3 months. This was characterized by weakly secretory endometrial glands with scarce or absent mitotic activity, variable stromal changes ranging from stromal compaction to focal pre-decidual reaction and thickening of the walls of spiral arterioles. These unusual morphological appearances were further referred to as 'non-physiologic secretory effects' or 'endometrial SPRM effects' (Williams *et al.*, 2007).

The clinical and histological effects of asoprisnil described above have been demonstrated in the presence of follicular phase estrogen concentrations (Wilkens *et al.*, 2008). Concern has previously been expressed that administration of a compound with progesterone antagonistic activity may leave the endometrium at risk of hyperplastic or even malignant changes due to exposure to unopposed estrogen. Previous studies have reported features of endometrial hyperplasia following administration of the progesterone antagonist mifepristone (Eisinger *et al.*, 2003). However, hyperplasia was not observed with use of low doses of 2 or 5 mg mifepristone, which after administration to 90 women for 120 days resulted in suppression of endometrial proliferation marker expression (Ki-67 and mitotic index) (Baird *et al.*, 2003). No study or case report has described complex hyperplasia or cytological atypia. No endometrial hyperplasia or evidence of unopposed estrogen effects have been described with asoprisnil after treatment for up to 3 months (Chwalisz *et al.*, 2005a, 2007).

Endometrial proliferation can be assessed in full thickness endometrial biopsies by means of Ki-67 and PH3 immunohistochemistry. The nuclear antigen Ki-67 can be detected in all phases of the replicating cell (G1, S, G2 and M) representing the growth fraction of a cell population (Gerdes *et al.*, 1984; Endl and Gerdes, 2000). More recently, Ki-67 has been described to play a role in ribosomal RNA synthesis as well as mitosis and may therefore also be expressed in non-proliferating cells (Bullwinkel *et al.*, 2006). The Ki-67 index is less specific than the mitosis-specific marker PH3, which is only expressed during the actual phase of mitosis (M) (Brenner *et al.*, 2003). When proliferation of normal endometrium in different phases of the menstrual cycle is assessed using various proliferation markers, they all show a high proliferation index during the proliferative phase with a significant decrease in the secretory phase. There is a high correlation between the direct mitotic count and the PH3 count, while the Ki-67 index shows the same trend but is less specific (Brenner *et al.*, 2003).

PTEN (phosphatase and tensin homologue) expression in endometrial tissue can be studied as a marker of early carcinogenesis. PTEN is a tumour suppressor gene product, which has been described as a gatekeeper for initiation of carcinogenesis in the endometrium (Mutter, 2001; Daikoku *et al.*, 2008). Loss of PTEN function has been demonstrated to occur as an early event in endometrial carcinogenesis and has therefore been suggested as a biomarker for premalignant disease even in histologically normal endometrium (Mutter *et al.*, 2000, 2001). The PTEN tumour suppressor gene is inactivated in up to 83% of endometrioid endometrial adenocarcinomas, the most common form of endometrial cancer, which has also been associated with the risk factor of exposure to unopposed estrogen (Mutter, 2001). It has been shown that exogenous hormones can alter PTEN expression. Progesterone has been shown to play an important role in eliminating PTEN-deficient endometrial cells when administered via a progestin-impregnated intrauterine device (Orbo *et al.*, 2006) or systemically (Zheng *et al.*, 2004). A compound with partial progesterone antagonist activity may raise the concern of an unfavourable effect on PTEN expression and therefore on the potential to influence the predisposition to latent endometrial precancerous lesions.

The objective of this study, which is a continuation of the above-mentioned study (Williams *et al.*, 2007), was to investigate in detail the effects of asoprisnil on proliferation of human endometrial, myometrial and leiomyoma tissues and on endometrial PTEN expression.

Materials and Methods

Study design

This was a Phase II multi-centre, double-blind, randomized, placebo-controlled study of asoprisnil administered for 12 weeks. The study group was composed of 33 premenopausal women from four centres (Edinburgh, Southampton, Glasgow, Liverpool) in good general health with symptomatic uterine fibroids, who were scheduled for hysterectomy mostly due to heavy menstrual bleeding (Williams *et al.*, 2007; Wilkens *et al.*, 2008). Each subject had at least one intramural, non-pedunculated submucosal or subserosal fibroid with a diameter of at least 2 cm confirmed by ultrasonography. Other inclusion criteria included age over 18 years, regular menstrual cycles of 17–42 days, negative pregnancy test, serum FSH of <30 mIU/ml, a minimum washout period of 2 months for oral contraceptive users, agreement to use double-barrier method of contraception throughout the study until hysterectomy and a normal cervical smear. All subjects were required to have a normal endometrial biopsy report based on an adequate specimen taken within 3 months of screening for entry into the study. The study was performed according to the ethical principles of the Declaration of Helsinki (1989 revision). The Institutional Review Board (Multicentre Research Ethics Committee) approved the protocol. All subjects voluntarily signed a full informed consent form.

Subjects were randomly assigned to one of three parallel treatment groups in a 1:1:1 ratio to receive daily doses of asoprisnil 10, 25 mg or placebo. Asoprisnil or placebo capsules were supplied in blister cards of identical appearance packaged in sealed kits. The drug was self administered as a single oral dose taken once daily. Treatment was initiated no later than the fifth day of the subject's menstrual cycle and continued for at least 12 weeks. Hysterectomy was performed within 24 h after

the final dose of drug. Blood specimens for determination of estradiol (E_2) and progesterone were collected within 24 h prior to hysterectomy. Compliance was monitored by the subjects returning all used and unused study medication blister cards.

Sample collection

After removal of the uterus in the operating theatre, the unfixed specimen was placed on ice and taken without delay to the local pathology laboratory. The specimen was oriented and a probe inserted through the external os of the cervix to define the position of the cavity. The uterus was opened using a long-bladed knife along the plane of the probe. The opened specimen was then placed in an adequate volume of 10% buffered formaldehyde and allowed to fix overnight. For large specimens, parallel parasagittal slices 2 cm in thickness were made to permit adequate fixation. The following day, the pathologist sampled the specimen for routine diagnostic reporting and took additional blocks for study assessment. Study blocks included full thickness endometrium with underlying myometrium. Leiomyomata were also sampled. All study samples were processed by routine methods for paraffin wax, and 5 μm sections prepared. Endometrial assessment was carried out by microscopic examination of haematoxylin–eosin stained sections. Phase of cycle was assessed using the updated conventional descriptive Noyes criteria of the normal menstrual cycle as described in Blaustein's Pathology of the Female Genital Tract (Mutter and Ferenczy, 2002).

Immunohistochemistry

Five-micrometre paraffin sections were de-waxed in Histoclear (National Diagnostics, Atlanta, GA, USA) for 10 min before rehydration in descending grades of alcohol. The slides were washed with 0.01 M phosphate-buffered saline (PBS; pH 7.4; Sigma) and pressure-cooked in 0.01 M sodium citrate (pH 6) for 5 min at setting 2/high (Tefal, Clipso, Nottingham, UK) for antigen retrieval. The tissue sections were cooled for 20 min and then washed again in PBS before blocking endogenous peroxidase activity by immersion in 3% hydrogen peroxide for 10 min at room temperature. After washing in PBS, the subsequent protocol differed for the two proliferation markers Ki-67 and PH3.

Ki-67 immunohistochemistry

Slides were incubated in non-immune horse serum (NHS; Vector Laboratories Inc, UK) in PBS for 20 min at room temperature in order to block non-specific binding of the primary antibody. The primary antibody Ki-67 Novocastra NCL-Ki67-MM1 (Novocastra, Newcastle-upon-Tyne, UK; 1:100 dilution in NHS/PBS) was added and the slides incubated for 30 min at 37°C. For the negative controls, the primary antibody was replaced with non-immune mouse immunoglobulin (Ig)G1 antibody at a matched antibody concentration to the Ki-67 antibody (1:1000). Subsequently, the sections were washed in PBS with added Tween 20 (PBST) before incubating in biotinylated horse anti-mouse antibody (Vector Laboratories) for 30 min at room temperature. Following another wash in PBST, an avidin–biotin–peroxidase complex (ABC-HRP; Vectorstain Laboratories) was applied for 30 min at room temperature before the final wash with PBST.

PH3 immunohistochemistry

At room temperature, sections were incubated in avidin (Vector Laboratories) for 15 min, rinsed in PBS and then incubated in biotin (Vector Laboratories) for a further 15 min. To block non-specific binding of the primary antibody, slides were incubated in non-immune goat serum (NGS; Autogen Bioclear Cat# 7) in PBS with 5% bovine serum albumin (BSA; Sigma Cat# A-7888) for 20 min at room temperature. The primary antibody PH3 (Cat# 06–570, Upstate Biotechnology, Buckingham, UK; 1:1000 dilution in NGS/PBS/BSA) was added and the slides incubated overnight at room temperature. For the negative controls, the primary antibody was replaced with non-immune rabbit antibody IgG at a matched antibody concentration to H3 (1:1000). Sections were washed in PBST before incubating in anti-rabbit envision kit (EnVision+ System-HRP, DAKO Cytomation) for 30 min at room temperature and washing again with PBST.

After the final wash with PBST, the protocols for both proliferation markers Ki-67 and PH3 were then followed by the addition of the chromagen 3, 3′-diaminobenzidine (DAKO). The reaction was stopped with distilled water when nuclear staining was detected by inspection under the microscope. Harris's haematoxylin was used for counterstaining. The sections were then dehydrated and finally mounted with Pertex (Cellpath plc, Hemel Hempstead, UK).

PTEN immunohistochemistry

For PTEN immunohistochemistry, the paraffin sections were de-waxed, rehydrated and pressure-cooked as described earlier. After cooling down for 20 min, the sections were transferred to the Bond-X immunostaining machine and processed using the Bond Refine Polymer Detection kit (Cat. No DS9800 Vision BioSystems Bond™, Newcastle-upon-Tyne, UK). The primary antibody was PTEN (NCL-PTEN, Novocastra; 1:600 dilution in Bond Antibody diluent, Vision BioSystems) and IgG from the same species and at the same concentration was used as the negative control (Mouse IgG_1 kappa, Sigma). Sections were dehydrated and mounted as described earlier.

Scoring

In myometrium and leiomyoma tissue, Ki-67 and PH3 immunostaining was assessed by randomly selecting 10 fields at ×250 magnification and counting all stained cells. To quantify the amount of staining in endometrium, stereological methods were applied and varied for assessment of Ki-67 and PH3, respectively. For assessment of PTEN immunostaining in endometrium, a histoscore was applied by two independent observers as previously described (Aasmundstad *et al.*, 1992). A separate histoscore was applied to surface epithelium, glandular epithelium, stroma, perivascular cells and endothelium, respectively.

Ki-67

Inspection of the endometrial tissue after immunostaining for Ki-67 gave the impression of scanty staining in the majority of sections. In order to quantify the level of immunoreactivity, stereological methods were used as previously described (Mahood *et al.*, 2005). The program used was Image-Pro plus 4.5.1 with Stereology-Pro 5.0 plug-in software (Media Cybernetics UK, Wokingham, Berkshire, UK) in combination with an Olympus BH-2 microscope fitted with a Prior automatic stage (Prior Scientific Instruments Ltd, Cambridge, UK). With the aid of the software, random fields were selected for counting and grids were placed over the fields at ×250 magnification. All 432 intersections of a grid were defined as points, and all points falling over tissue were counted as one of the following categories: (a) unstained epithelial cell, (b) stained epithelial cell, (c) unstained stromal cell, (d) stained stromal cell and (e) lumen (i.e. the empty space within glands or vessels). The proportion of tissue occupied by each of these categories was expressed as a percentage of total points counted.

The number of fields counted was dependent on obtaining a percentage SE value of $<10\%$. For all but four tissue sections, 10 fields (i.e. 4320 points) were sufficient to obtain a percentage SE of $<10\%$ for the categories of unstained cells and lumen. For three of the remaining tissue sections, 50 fields (i.e. 21 600 points) and in one case 20 fields

(i.e. 8640 points) were counted. The number of points occupied by stained epithelial or stromal cells was counted in a total of 50 fields for all tissue sections. According to the statistical formula for sample size calculations, 50 fields (or 21 600 points) are sufficient to detect a proportion of 0.01% of stained cells assuming a null hypothesis of 0.00001%. Therefore, the point count was limited to 50 fields for all tissue sections (Kirkwood and Sterne, 2003).

PH3

Inspection of the endometrial tissue after staining for PH3 revealed very scanty immunostaining. In order to quantify the positive immunoreactivity in the tissue sections, the total number of stained epithelial and stromal cells in all sections was counted. Stereological methods were used to determine the area of endometrium in each section. The program used was Image-Pro plus 4.5.1 with Stereology-Pro 5.0 plug-in software (Media Cybernetics UK) in combination with an Olympus BH-2 microscope fitted with a Prior automatic stage (Prior Scientific Instruments Ltd). The cell count was expressed as the number of stained cells per mm^2 endometrium.

Statistical analysis

For statistical analysis, each group of asoprisnil-treated subjects (10 and 25 mg) was compared with a subgroup of placebo-treated subjects who had undergone hysterectomy in the secretory phase of their menstrual cycle. The Wilcoxon's rank sum test was used for this analysis and significance was determined at 0.05 level using Hochberg's multiple comparison procedure.

Results

A total of 33 patients were included in the study. Placebo and asoprisnil groups had a well-matched distribution for age, weight and height (Table I). Ten subjects had been treated with placebo, 12 had received 10 mg asoprisnil and 11 had received 25 mg asoprisnil for an average of 95 days. There was satisfactory drug compliance in all groups. Two placebo-treated subjects had undergone hysterectomy in the proliferative phase of their menstrual cycle and were excluded from the statistical analysis. The remaining eight placebo-treated subjects had been in the secretory phase of their cycle and constituted the subgroup for comparison to the asoprisnil-treated groups.

Proliferation marker expression was low in all asoprisnil-treated subjects as well as placebo-treated subjects in the secretory phase in all uterine tissues. There was high proliferation marker expression in the endometrium of the two placebo-treated subjects in the proliferative phase of the menstrual cycle.

Expression of Ki-67 and PH3 in endometrium

Assessment of proliferation marker expression in endometrial stroma is suggestive of a suppressive effect of asoprisnil (Fig. 1A). Following the method of quantifying Ki-67 immunostaining as described earlier, the median of grid points occupied by stained cells in endometrial epithelium and stroma was <2% in specimens from both asoprisnil- and placebo-treated subjects. Following asoprisnil administration, there was a statistically significant and dose-dependent decrease in Ki-67 expression in endometrial stroma (Fig. 1A) compared with the placebo-treated subjects in the secretory phase of their menstrual cycle. There was no statistically significant difference between treatment groups in Ki-67 expression in endometrial glandular epithelium (Fig. 1B). The median of counted stained endometrial cells following immunostaining for PH3 was generally very low in epithelium and stroma of both asoprisnil- and secretory phase placebo-treated subjects (<3/mm^2). There was no statistically significant difference between treatment groups (Fig. 2). The PH3 data were normalized to the total area of endometrium. The data were also assessed in relation to only epithelial or stromal cells, and those results were comparable and did not add to the results as presented.

Expression of Ki-67 and PH3 in myometrium and leiomyomata

There also appeared to be a suppressive effect of asoprisnil on myometrial proliferation. There was very low PH3 expression in both asoprisnil- and secretory phase placebo-treated groups (median of less than 10 stained cells per 10 fields at ×250 magnification), and the differences were not statistically significant. There was however a dose-dependent decrease in Ki-67 expression in both asoprisnil-treated groups compared with the secretory phase placebo-treated subjects, but only the difference between asoprisnil 25 mg and secretory phase placebo reached statistical significance (Table II).

Table I Demographic data for patients with symptomatic uterine leiomyomata given placebo or asoprisnil daily for 12 weeks before hysterectomy

Variable	Treatment group			
	Placebo (n = 10)	Asoprisnil 10 mg (n = 12)	Asoprisnil 25 mg (n = 11)	All subjects (n = 33)
Age (years)				
Mean (SD)	41.8 (3.6)	45.1 (3.5)	44.6 (6.0)	43.9 (4.6)
Min-Max	37–48	39–50	35–52	35–52
Weight (kg)				
Mean (SD)	73.4 (11.7)	73.8 (17.7)	75.9 (11.8)	74.4 (13.8)
Min-Max	54–89	45–105	60–96	45–105
Height (cm)				
Mean (SD)	165.3 (6.4)	164.3 (4.7)	165.6 (7.3)	165.1 (6.0)
Min-Max	158–177	156–172	157–178	156–178

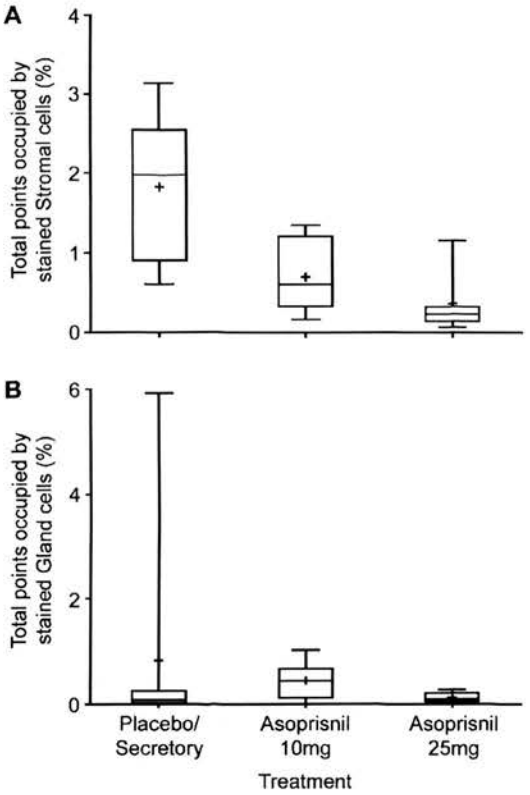

Figure 1 Ki-67 expression in endometrial stroma (**A**) and glands (**B**) following administration of asoprisnil.

Ki-67 expression is quantified by percentage of grid points overlying a stained glandular or stromal cell respectively, following the stereological projection of a grid over several views of a tissue section. In the box plots, the box spans between lower and upper quartiles with the horizontal line within it representing the median; the whiskers extend to the minimum and maximum observations; the cross represents the mean. Each asoprisnil group is compared with placebo (secretory phase) using Wilcoxon's rank sum test. In stroma, both asoprisnil groups have a statistically significantly lower expression of Ki-67 than placebo using Hochberg's multiple comparison procedure at 0.05 level; in glands, neither difference between an asoprisnil group and placebo is statistically significant.

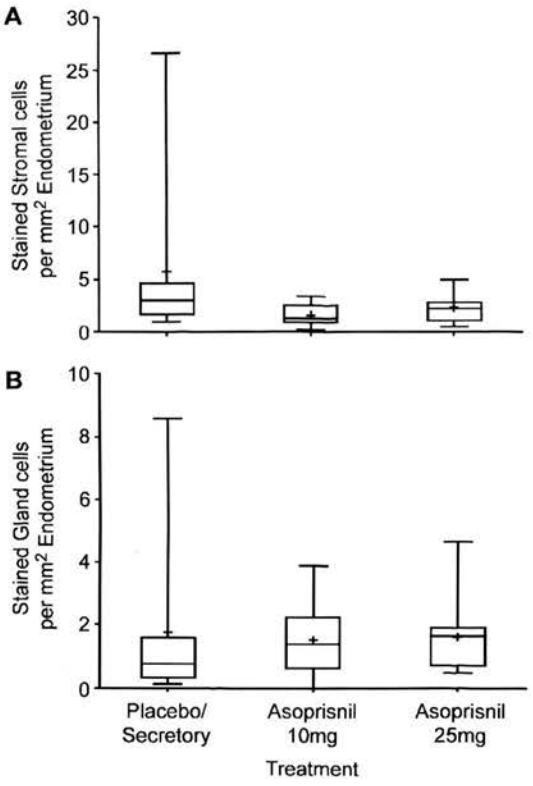

Figure 2 PH3 expression in endometrial stroma (**A**) and glands (**B**) following administration of asoprisnil.

PH3 expression is quantified as stained glandular or stromal cells per mm^2 endometrium following a count of all stained cells in the available tissue section and stereological measurement of the endometrial area. Each asoprisnil group is compared with placebo (secretory phase) using Wilcoxon's rank sum test. None of the differences between an asoprisnil group and placebo are statistically significant. PH3, anti-phospho-histone H3.

Assessment of Ki-67 and PH3 expression in leiomyoma tissue revealed a similar trend. There was very low PH3 expression in tissue sections from asoprisnil- and placebo-treated subjects, and the differences between groups were not statistically significant. There was an apparent reduction in Ki-67 expression in both asoprisnil-treated groups, but the differences did not reach statistical significance (Table III).

Endometrial expression of PTEN

There was no difference in immunohistochemical detection of PTEN expression between treatment groups in any of the tissue compartments assessed. PTEN immunhistochemistry resulted in a nuclear staining pattern with similar intensity in glandular epithelium and stroma (Fig. 3). The staining intensity was strongest in the surface epithelium (median histoscores of 125–180) and only weak in endothelium and perivascular cells (median histoscores of 25–32.5 for perivascular cells and 30–45 for endothelium). In asoprisnil and placebo groups, there were inter-individual variations independent of the treatment received with some subjects showing very weak and others quite strong staining. However, there was no significant difference between treatment groups (Fig. 4).

Blood concentrations of E_2 and progesterone

E_2 and progesterone levels were measured within 24 h prior to hysterectomy (Table IV). The mean E_2 value was lower in the 25-mg asoprisnil group compared with both placebo and 10-mg asoprisnil groups. Mean progesterone values were substantially lower in both asoprisnil-treated groups compared with placebo. Although variability across the groups was high, the median progesterone values revealed the same pattern.

Discussion

This study shows that asoprisnil administered daily for 12 weeks exerts an inhibitory effect on endometrial proliferation and proliferation of myometrium and leiomyomata.

Table II Proliferation marker expression in myometrium by immunohistochemistry

	Placebo/secretory	Asoprisnil 10 mg		Asoprisnil 25 mg	
	Median	Median	*P*-value	Median	*P*-value
Ki-67: count of stained cells per 10 fields at ×250	80.0	31.5	0.558	19.0	0.024$
PH3: count of stained cells per 10 fields at ×250	8.0	3.5	0.099	5.0	0.582

Each asoprisnil group is compared with placebo using Wilcoxon's rank sum test; $ denotes statistical significance at 0.05 level using Hochberg's multiple comparison procedure. PH3, anti-phospho-histone H3.

Table III Proliferation marker expression in leiomyoma by immunohistochemistry

	Placebo/secretory	Asoprisnil 10 mg		Asoprisnil 25 mg	
	Median	Median	*P*-value	Median	*P*-value
Ki-67: count of stained cells per 10 fields at ×250	237.0	66.0	0.078	86.0	0.088
PH3: count of stained cells per 10 fields at ×250	5.5	3.0	0.355	4.0	0.707

Each asoprisnil group is compared with placebo using Wilcoxon's rank sum test.

Within the placebo group in this study, endometrial glands and stroma showed high proliferation marker expression in subjects who had undergone hysterectomy in the proliferative phase of their menstrual cycle compared with low expression in subjects in the secretory phase, as previously reported (Brenner *et al.*, 2003). Proliferation marker expression following 12 weeks of treatment with asoprisnil was low in most uterine tissues and comparable to the low level of proliferation observed in the placebo-treated subjects in the secretory phase. There was no difference in PH3 expression between treatment groups in endometrium, myometrium or leiomyoma tissue, a likely reflection of the very scanty immunostaining. Ki-67 expression in endometrial epithelium was not significantly different in asoprisnil-treated subjects compared with placebo-treated subjects in the secretory phase. Asoprisnil had a dose-dependent inhibitory effect on proliferation in endometrial stroma as indicated by decreased Ki-67 expression. There was no evidence of altered endometrial PTEN expression by administration of asoprisnil. Dose-dependent suppression of proliferation was demonstrated similarly in myometrium but was only significant with the higher dose of asoprisnil. Proliferation of leiomyoma tissue appeared to be inhibited by asoprisnil even though the differences did not reach statistical significance.

The endometrial antiproliferative effect of asoprisnil demonstrated in this study is consistent with previous clinical trial reports. Proliferation marker expression was suppressed or unchanged compared with the secretory menstrual cycle phase. In a previous trial, various doses of asoprisnil (5 mg once daily to 50 mg twice daily) were administered to healthy premenopausal women for 28 days. In endometrial biopsies collected following treatment (with a Pipelle® endometrial sampler), there was no evidence of endometrial hyperplasia or other appearances suggestive of an unopposed estrogen effect. Importantly, there was a common finding of a unique endometrial appearance, which has since been classified as 'non-physiologic secretory effect' and which has not been previously described with any hormonally active agent (Chwalisz *et al.*, 2005a). This appearance, now referred to as 'SPRM endometrial effect', seems to reflect the mixed progesterone agonistic/antagonistic properties of asoprisnil in the human endometrium.

In a further trial with administration of 5, 10 or 25 mg asoprisnil to women with uterine leiomyomata for 12 weeks, Pipelle® endometrial biopsies were also obtained following treatment. Histological assessment revealed similar findings. There was no case of endometrial hyperplasia or cytological atypia. A majority of endometrial samples following asoprisnil treatment showed distinct changes consistent with the 'SPRM endometrial effect' (Chwalisz *et al.*, 2007). In a 39-week toxicity study with asoprisnil in adult cynomolgus monkeys, the endometrium appeared atrophic with suppressed gland proliferation and stromal compaction. Some glands appeared dilated and cystic in the absence of any hyperplastic features (DeManno *et al.*, 2003). However, this study neither showed secretory changes in endometrial glands nor thickening of the wall of spiral arterioles.

Studies in non-human primate models have been crucial in the discovery and development of SPRMs and have provided valuable insight into their possible mechanisms of action (Chwalisz *et al.*, 2006). Endometria from monkeys as well as humans have shown a distinct morphological response following exposure to SPRMs pointing to an antiproliferative effect (Wolf *et al.*, 1989). However, comparisons between monkeys and humans have also indicated some important differences highlighting the need for caution when extrapolating results from non-human primate studies to the human. This may be due to differences in the steroid receptor pharmacology of the monkey and human endometrium (Chwalisz *et al.*, 2006). It is possible that the balance between agonist and antagonist effect of asoprisnil is in favour of the agonist side in humans. This could result from species differences in metabolic end-products or from species-specific cellular environments creating a different balance of coactivator and corepressor expressions (Chwalisz *et al.*, 2008). The findings of secretory

endometrial gland changes and formation of thick-walled endometrial spiral arteries following treatment with asoprisnil were unique to the human endometrium and had not previously been observed in the monkeys. Suppression of proliferation marker expression due to asoprisnil exposure was common to both human and non-human endometria. Endometrial Ki-67 and PH3 expression was suppressed in cynomolgus monkeys treated with 10, 30 or 90 mg/kg asoprisnil for 90 days. The suppression was significant when compared with samples from the placebo group in the proliferative cycle phase (Brenner *et al.*, 2005). There was no significant difference between asoprisnil-treated monkeys and monkeys in the secretory cycle phase corresponding to the findings in this study. There was no differentiation into separate tissue compartments (stroma and epithelium) in the monkey studies, while this study showed that in the human Ki-67 expression particularly in endometrial stroma is significantly suppressed after 3 months asoprisnil treatment.

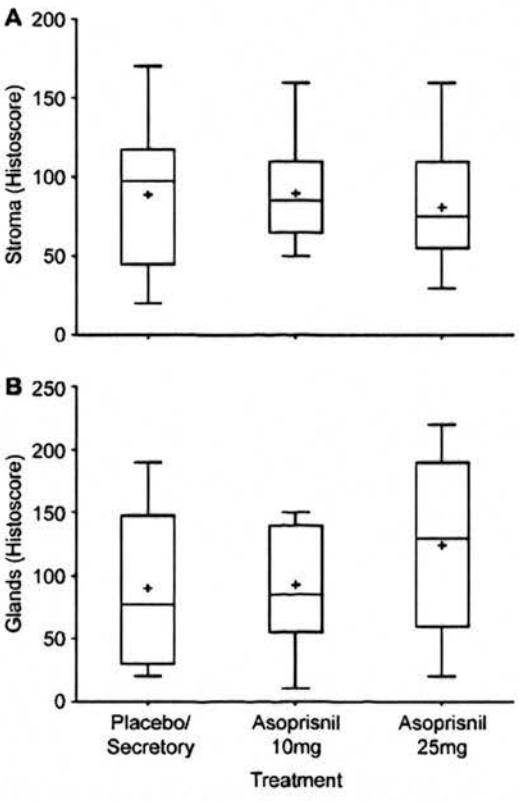

Figure 3 PTEN expression in endometrial stroma (**A**) and glandular epithelium (**B**) following administration of asoprisnil.

PTEN expression is quantified by applying a histoscore (0–300). Each asoprisnil group is compared with placebo (secretory phase) using Wilcoxon's rank sum test. None of the differences between an asoprisnil group and placebo are statistically significant. PTEN, phosphatase and tensin homologue.

The exact mechanism of action of the endometrial antiproliferative effect of SPRMs has still not been fully elucidated. It has been suggested that the endometrial effects of asoprisnil are mediated by the endometrial vasculature and in particular the spiral arteries (Chwalisz *et al.*, 2000), which may be targeted via the perivascular cells (Chwalisz *et al.*, 2006). This hypothesis appears to be supported by the striking effect of asoprisnil on the formation of thick-walled spiral arterioles in humans. However, the absence of similar morphological vascular changes in non-human primate models suggests there may be another pathway, and more recently the role of the endometrial androgen receptor (AR) has been emphasized as a potential mechanism of the endometrial antiproliferative effect (Brenner *et al.*, 2002; Brenner and Slayden, 2005). Androgens are known to inhibit estrogenic effects in the primate endometrium, and AR has been shown to be up-regulated by treatment with various progesterone antagonists and SPRMs in monkeys and mifepristone in humans (Brenner *et al.*, 2002, 2005). Moreover, it was possible to suppress the endometrial antiproliferative effect induced by progesterone antagonists by adding the AR antagonist flutamide (Slayden and Brenner, 2003). These findings imply that a functional AR is required for SPRMs to exert their antiproliferative effect on the endometrium.

Previous *in vitro* studies have suggested asoprisnil to have a cell type-specific antiproliferative effect on uterine leiomyoma cells compared with normal myometrial cells (Chen *et al.*, 2006). A direct effect of asoprisnil on leiomyoma cells may result in suppressed growth and therefore reduced fibroid volume as previously reported (Chwalisz *et al.*, 2007). The concomitant suppression of myometrial proliferation observed in this study suggests there may be an additional mechanism of action, for example, an impact on uterine artery blood flow. Both

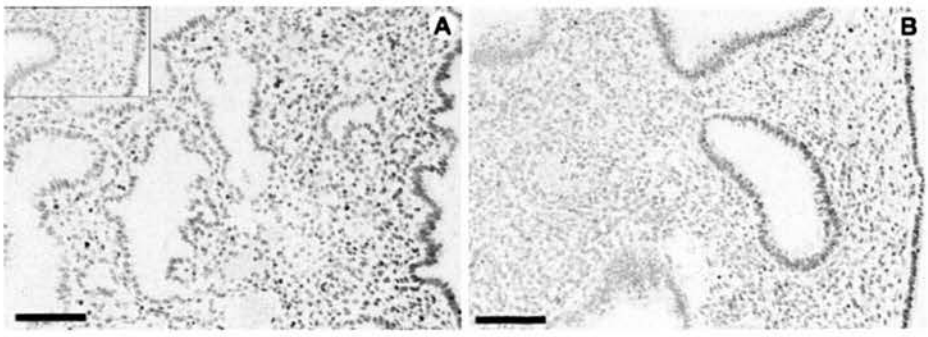

Figure 4 PTEN immunolocalization in endometrium; scale bar 100 μm.

Placebo group in secretory cycle phase; inset—negative control (**A**). Treatment group asoprisnil 25 mg (**B**).

Table IV Blood E_2 and progesterone concentrations within 24 h of hysterectomy

	Treatment group		
	Placebo (n = 10)	Asoprisnil 10 mg (n = 12)	Asoprisnil 25 mg (n = 11)
Estradiol (ng/dl), mean ± SD	13.4 ± 9.1	13.2 ± 8.3	5.0 ± 4.2
Progesterone (ng/dl)			
Mean ± SD	759.5 ± 689.2	354.8 ± 503.3	56.5 ± 78.4
Median	596	43	23

asoprisnil (Wilkens *et al.*, 2008) and mifepristone (Reinsch *et al.*, 1994) have been shown to reduce uterine artery blood flow.

This study has demonstrated that endometrial PTEN expression is not altered by administration of asoprisnil. This finding, together with the low endometrial Ki-67 and PH3 expression after 3 months treatment, supports the conclusion that the effect of asoprisnil on endometrium is antiproliferative and does not promote carcinogenesis. It justifies the assumption that the feature of cystically dilated glands occasionally found in asoprisnil-treated endometria is not associated with hyperplasia and is not pre-malignant.

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Effects of the Selective Progesterone Receptor Modulator Asoprisnil on Uterine Artery Blood Flow, Ovarian Activity, and Clinical Symptoms in Patients with Uterine Leiomyomata Scheduled for Hysterectomy

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Introduction: Asoprisnil, a novel orally active selective progesterone receptor modulator, is being studied for the management of symptomatic uterine leiomyomata. The exact mechanism of action is not yet discerned. The primary objectives of this double-blind, randomized, placebo-controlled study included evaluation of the effect of asoprisnil on uterine artery blood flow. Furthermore, we assessed effects of asoprisnil on leiomyoma symptoms.

Patients and Methods: Thirty-three premenopausal patients scheduled for hysterectomy due to symptomatic uterine leiomyomata were recruited in four centers and treated with 10 or 25 mg asoprisnil or placebo for 12 wk before surgery. At baseline and before hysterectomy, all patients underwent sonographic assessment to measure impedance to uterine artery blood flow, determined by resistance index and pulsatility index, as well as volumes of largest leiomyoma and uterus. In addition, patients recorded intensity and frequency of menstrual bleeding on a menstrual pictogram. Each asoprisnil treatment was compared with placebo.

Results: The increased pulsatility index in both asoprisnil groups and the statistically significantly increased resistance index within the 25-mg asoprisnil group suggest a moderately decreased uterine artery blood flow. Analysis of menstrual pictogram scores showed a statistically significant larger decrease in frequency and intensity of bleeding for both asoprisnil groups compared with placebo. Bleeding was suppressed by asoprisnil 25mg in 91% of patients. Asoprisnil treatment was well tolerated when administered daily for a 12-wk period, and no serious adverse events occurred.

Conclusion: Asoprisnil moderately reduced uterine artery blood flow. This effect may contribute in part to the clinical effects of asoprisnil. ***(J Clin Endocrinol Metab* 93: 4664–4671, 2008)**

Uterine leiomyomata are benign smooth muscle tumors originating from the myometrium. They are present in up to 70% of women even though asymptomatic in over half of the cases with 20–25% of women of reproductive age clinically affected (1, 2). The commonest symptoms are heavy menstrual bleeding (HMB) and pressure symptoms. With currently limited options for medical therapy, uterine leiomyomata are the second most frequent indication for hysterectomy in the United Kingdom

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Abbreviations: ANCOVA, Analysis of covariance; AE, adverse event; E_1G, estrone glucuronide; ELA, evidence of luteal activity; HMB, heavy menstrual bleeding; MP, menstrual pictogram; NELA, no evidence of luteal activity; NSAID, nonsteroidal antiinflammatory drug; PdG, pregnanediol glucuronide; PI, pulsatility index; RI, resistance index; UFS-QOL, Uterine fibroid symptom and health-related quality-of-life.

(3, 4). In the United States, 600,000 hysterectomies are performed annually with HMB as the most common indication (5).

There is growing evidence that progesterone and the progesterone receptor play a key role in fibroid growth and development. Contrary to previous understanding that leiomyoma growth is mainly estrogen related, recent data from clinical and *in vitro* studies indicate that progesterone plays a pivotal role (6, 7). Some clinical studies have shown that synthetic progestins reverse the effect of GnRH agonists on leiomyoma volume, which indirectly indicates the effects of GnRH agonists on leiomyomata may be due partly to cessation of progesterone secretion (8). Furthermore, a reduction in mean leiomyoma volume was demonstrated in small, uncontrolled, clinical studies with the progesterone receptor antagonist mifepristone (9). Mifepristone has been shown to reduce uterine artery blood flow in patients with uterine leiomyomata (10). Collectively, these data suggest that progesterone may have a stimulatory effect on leiomyoma growth.

Asoprisnil (J867) is a novel, orally active and selective progesterone receptor modulator (SPRM), which exhibits partial and mixed agonist and antagonist effects on various progesterone target tissues in animals and humans (11–13). Asoprisnil exhibits endometrial antiproliferative effects in nonhuman primates in the presence of follicular-phase estradiol levels (11, 14).

The effects of 3 months treatment with asoprisnil in women with uterine leiomyomata have been reported. Asoprisnil suppressed uterine bleeding in 28, 64, and 83% of subjects at 5, 10, and 25 mg, respectively, and reduced leiomyoma and uterine volumes (15).

The current study was designed to evaluate the mechanism of action of asoprisnil in patients with symptomatic leiomyomata scheduled for hysterectomy. The primary objectives were to assess the effects of asoprisnil on uterine artery blood flow through measurements of impedance (resistance and pulsatility indices). The effects on endometrial, myometrial, and leiomyomata morphology, have been reported elsewhere (16). Furthermore, we investigated effects of asoprisnil on leiomyoma symptoms, including semiquantitative assessment of uterine bleeding using a menstrual pictogram (MP), and ovarian activity.

Subjects and Methods

Women studied

Premenopausal women were recruited from four centers (Edinburgh, Southampton, Glasgow, and Liverpool). All subjects were in good health and scheduled for hysterectomy due to symptomatic uterine leiomyomata, mostly due to HMB. Each patient had at least one leiomyoma (diameter $\geq$ 2 cm) or multiple small leiomyomata (uterine volume $\geq$ 200 cm^3) confirmed by ultrasonography. In all cases, the clinical decision for hysterectomy was taken before recruitment. Inclusion and exclusion criteria were applied as previously described (16). Patients were required to have a washout period of 2–12 months for hormonal medications before screening. Non-steroidal anti-inflammatory drugs (NSAIDs) and tranexamic acid were permitted during screening and treatment periods. All patients provided informed consent. The study protocol was approved by the Multicenter Research Ethics Committee.

Study design

This was a phase II, multicenter, randomized, double-blind, placebo-controlled study of asoprisnil administered to patients with symptomatic uterine leiomyomata for 12 wk. Dose selection was based on previous phase I and II studies. A treatment regime with doses of 10 and 25 mg asoprisnil for a duration of 12 wk had been shown to effectively suppress uterine bleeding and reduce leiomyoma and uterine volumes while being safe and well tolerated (15).

Screening procedures and enrollment were carried out as previously described (16). Subjects in three parallel dose groups in a 1:1:1 ratio received once-daily oral doses of asoprisnil of 10 or 25 mg asoprisnil or placebo. They and all study personnel were blinded to treatment groups. Treatment was initiated no later than the fifth day of the patient's menstrual cycle and continued for 12 wk until hysterectomy. Hysterectomy was performed within 24 h of the final dose. Throughout the study, each patient was closely monitored for occurrence of adverse events (AEs) and standard laboratory safety parameters.

Sonographic assessment

Color Doppler imaging by transvaginal ultrasound was employed to determine blood flow of the uterine arteries before the first study drug dose and after 12 wk. Blood flow was estimated using two impedance indices: resistance index (RI) and pulsatility index (PI) defined as follows: RI = systolic − end diastolic peak velocity/systolic peak velocity; PI = systolic − end diastolic peak velocity/time-averaged maximum velocity (17). For each impedance index, two measurements were taken from left and right arteries, respectively; each side's index was calculated using the mean of the two, and further analyses used the mean of both sides (18). Study sites used the same color Doppler imaging methods. Scans were performed by the same ultrasonographer at each site. The largest leiomyoma and uterus were measured and the volumes estimated using the volume of an ellipsoid. The position of the fibroids within the uterus was not specifically recorded further to previous evidence that symptoms of HMB do not appear to correlate with fibroid location (19).

MP

At screening, patients were issued a MP in a daily diary to be kept throughout the study. Patients recorded daily any uterine bleeding. Whenever uterine bleeding exceeded spotting, the amount of blood loss was quantified and documented in the MP. Patients were supplied with standardized sanitary products. The MP scores, representing blood loss in milliliters, were calculated as described previously (20) and then summed for each patient for the last full menstrual cycle before randomization menses normalized to 28 d and for each 28-d treatment period, producing a total score for each subject for baseline, wk 1–4, wk 5–8, and wk 9–12. The number of days with bleeding was calculated from the diaries for the pretreatment cycle and the three 28-d treatment periods. To evaluate improvement in uterine bleeding, change of MP scores and of days with bleeding from baseline to each month and final month was calculated and summarized. The percentage of subjects with suppression of uterine bleeding during the treatment period was calculated for each treatment group.

Uterine fibroid symptom and health-related quality-of-life (UFS-QOL) questionnaire

Before commencing the study drug and before hysterectomy, patients completed the Uterine Fibroid Symptom and Health-Related Quality-of-Life questionnaire (UFS-QOL) (21) with its subscales of concern, effect on activities, energy/mood, control, self-consciousness, sexual function, and symptom severity.

Ovarian activity

Urine aliquots (first voided urine of the day) were collected twice weekly during screening and throughout the treatment period and frozen at −20 C for subsequent analysis. Ovarian activity was determined by assessing urinary pregnanediol glucuronide (PdG) and estrone glucuro-

nide (E_1G) levels, which were measured using ELISA. Hormone concentrations were corrected for creatinine excretion and expressed as ratios of the creatinine concentration to urine volume (22).

Evidence of luteal activity (ELA) *vs.* no evidence of luteal activity (NELA) was determined from urinary PdG levels using two algorithms (23); in the first algorithm, a PdG level was considered ELA if it was at least three times the minimum 3-concentration moving average of the past 4 wk; the second algorithm had an additional criterion that, to be considered ELA, a PdG level had to be at least 0.5 mmol/mol creatinine. For each 4-wk period and each treatment group, the percentage of patients with NELA and 95% exact confidence intervals were calculated.

Ovarian follicular activity during treatment was determined by comparing E_1G concentrations during the 12-wk treatment period to pretreatment follicular phase concentrations (baseline). Based on the method described by Brown *et al.* (24), ovarian activity was labeled as continued ($E_1G \geq 50\%$ above baseline on at least two occasions, separated by ≥13 d, with no E_1G concentrations ≥50% above baseline), partially suppressed (E_1G concentration ≥50% above baseline on at least one occasion while not meeting the definition of continued follicular activity), or totally suppressed ($E_1G < 50\%$ above the baseline throughout treatment period). Number and percentage of patients belonging to each category were calculated for each treatment group.

Data analysis and statistical methods

Comparison of each asoprisnil treatment with placebo was performed using pairwise comparisons within the framework of analysis of covariance (ANCOVA) models for assessments of change in RI, PI, and MP scores, number of days with bleeding, and UFS-QOL scores. The ANCOVA models for RI and PI included factors of treatment and investigator as fixed effects and baseline value as a covariate, whereas the models for MP scores, number of days with bleeding, and UFS-QOL scores included treatment as a factor and baseline value as a covariate. In addition, a paired *t* test was performed for RI and PI on the change from baseline to final visit for each treatment group. Percent change in volume of the largest leiomyoma and the uterus was compared between each asoprisnil group and placebo using Wilcoxon's rank sum test. Percentage of patients with suppression of uterine bleeding was compared by Fisher's exact test. For efficacy endpoints, Hochberg's multiple comparison method was applied to control for pairwise comparisons at a significance level of 0.05. No statistical inference was performed on safety variables.

The planned sample size for this study was 15 patients per treatment arm. This sample size would provide greater than 95% power to detect a 0.08 difference in RI between the asoprisnil and the placebo group using a two-tailed two-sample *t* test with a common SD of 0.05 (with a 0.05 significance level).

The study was closed with a total of 33 patients. With 11 patients per group and assumptions as above, the power to detect a 0.08 difference in RI was 94%.

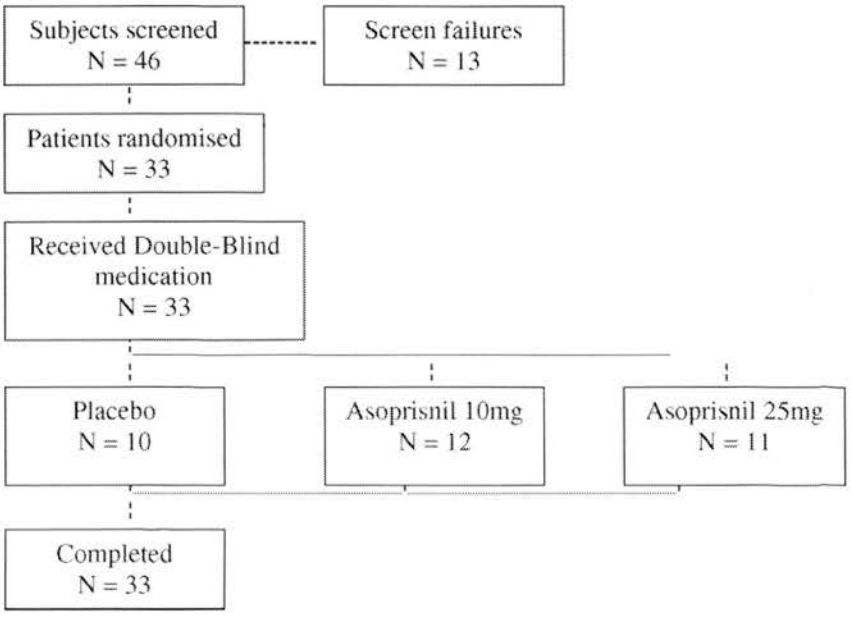

FIG. 1. Patient enrollment: numbers of patients at different stages of the clinical study.

Results

Patient demographics

Thirty-three patients were enrolled. Thirteen screen failures occurred. Ten, 12, and 11 patients received placebo and 10 and 25 mg asoprisnil, respectively. All 33 patients completed the study including 12 wk treatment, the scheduled hysterectomy, and follow-up after 6 wk (Fig. 1).

Treatment and placebo groups were well matched regarding race, age, height, and weight (Table 1). Drug compliance was satisfactory in all groups. No patients developed withdrawal criteria during the study or received the wrong treatment or an incorrect dose. Three patients (one on placebo and two on 25 mg asoprisnil) took tranexamic acid to control menstrual bleeding, but the median use per month was nil in each group. Median intake of NSAIDs was higher in the 10-mg asoprisnil group (1.2 d/month) than in the placebo or 25-mg asoprisnil groups (median of nil per month). During treatment, NSAIDs were primarily taken for headache, joint, or muscular pain and for dysmenorrhea. The differences between groups were not expected to influence study results.

Effects on uterine artery blood flow

Neither asoprisnil group had a change from baseline to final visit in RI that was statistically significantly different from placebo. There was, however, a statistically significant increase in RI from baseline to final visit within the 25-mg asoprisnil group, indicating decreased uterine artery blood flow (Table 2).

The PI increased statistically significantly from baseline to final visit in both asoprisnil groups compared with placebo, indicating decreased uterine artery blood flow. From baseline to final visit, the PI increased in the 25-mg asoprisnil group although unchanged after asoprisnil 10 mg with a statistically significant decrease within the placebo group (Table 2).

Effects on volume of largest leiomyoma and uterus

From baseline to final visit, the median percent change in largest leiomyoma volume showed a decrease after 25 mg asoprisnil (−25.8%) and a small increase in the placebo group (4.9%), with a very minor decrease after asoprisnil 10 mg (−0.4%). The differences between each asoprisnil group and placebo in percent change of largest leiomyoma volume or uterine volume were not statistically significant.

TABLE 1. Demographic data at baseline

Variable	Treatment group			
	Placebo (n = 10)	Asoprisnil 10 mg (n = 12)	Asoprisnil 25 mg (n = 11)	All subjects (n = 33)
Race, n (%)				
Black	1 (10)	2 (16.7)	1 (9.1)	4 (12.1)
Caucasian	9 (90)	10 (83.3)	10 (90.9)	29 (87.9)
Age (yr)				
Mean (SD)	41.8 (3.6)	45.1 (3.5)	44.6 (6.0)	43.9 (4.6)
Min-Max	37–48	39–50	35–52	35–52
Weight (kg)				
Mean (SD)	73.4 (11.7)	73.8 (17.7)	75.9 (11.8)	74.4 (13.8)
Min-Max	54–89	45–105	60–96	45–105
Height (cm)				
Mean (SD)	165.3 (6.4)	164.3 (4.7)	165.6 (7.3)	165.1 (6.0)
Min-Max	158–177	156–172	157–178	156–178

Race, age, weight, and height distribution across the three treatment groups. Max, Maximum; Min, minimum.

Effects on uterine bleeding

Treatment with asoprisnil led to a substantial decrease in uterine bleeding. There was a large mean reduction in blood loss in the final month compared with baseline in both asoprisnil groups, which was statistically significantly different from the mean increase in the placebo group (Table 3). These decreases were already apparent during the first 4 wk of treatment.

Patients treated with 10 and 25 mg asoprisnil had bleeding of 7.0 and 8.0 d on average at baseline, which decreased to 1.2 and 0.2 d in the final month, respectively. The placebo group had a mean number of 7.3 bleeding days at baseline and the final month. The difference between asoprisnil groups and placebo was statistically significant ($P < 0.001$). The decrease in the asoprisnil groups was evident during the first month and continued throughout the treatment period. Suppression of uterine bleeding was experienced by 33% of patients treated with 10 mg asoprisnil and 91% treated with 25 mg, compared with none of the patients in the placebo group. The difference between the 25-mg asoprisnil and placebo groups was statistically significant ($P < 0.001$).

UFS-QOL

Results of the UFS-QOL total score, and in particular the subscales of concern, activities, control, and self-consciousness, showed statistically significant improvement from baseline to final visit for both asoprisnil groups compared with placebo, indicating an effect on quality of life. Reduced symptom severity was observed in both asoprisnil groups but was statistically significant only with 25 mg asoprisnil compared with placebo (Fig. 2).

TABLE 2. Analysis of RI and PI

	Treatment group			Between-groups *P* value[a]	
	Placebo (n = 10) mean ± SD	Asoprisnil 10 mg (n = 12) mean ± SD	Asoprisnil 25 mg (n = 11) mean ± SD	Asoprisnil 10 mg *vs.* placebo	Asoprisnil 25 mg *vs.* placebo
RI					
Baseline	0.73 ± 0.10	0.76 ± 0.09	0.71 ± 0.08	NA	NA
Final visit	0.71 ± 0.17	0.75 ± 0.10	0.77 ± 0.08	NA	NA
Change from baseline	−0.02 ± 0.13	−0.01 ± 0.06	0.06 ± 0.08	0.756	0.146
Within-group *P* value[b] (change from baseline)	0.629	0.689	0.034[c]	NA	NA
PI					
Baseline	1.69 ± 0.60	1.80 ± 0.72	1.52 ± 0.44	NA	NA
Final visit	1.27 ± 0.33	1.81 ± 0.67	1.81 ± 0.48	NA	NA
Change from baseline	−0.42 ± 0.42	0.01 ± 0.56	0.30 ± 0.54	0.019[d]	0.005[d]
Within-group *P* value[b] (change from baseline)	0.012[c]	0.956	0.099	NA	NA

Mean changes of RI and PI (impedance indices to quantify uterine artery blood flow as determined by color Doppler imaging) from baseline to final visit in the three treatment groups (placebo, 10 mg asoprisnil, and 25 mg asoprisnil). NA, Not applicable.

[a] From ANCOVA model for change from baseline to final visit including fixed effects of treatment and investigator and baseline mean RI/PI as a covariate.

[b] A *t* test was performed on change from baseline to final visit for each treatment group.

[c] Statistical significance at 0.05 level.

[d] Statistical significance at 0.05 level using Hochberg's multiple-comparison procedure.

TABLE 3. MP scores

	Treatment group			*P* value[a]	
	Placebo (n = 10) mean ± SD	Asoprisnil 10 mg (n = 12) mean ± SD	Asoprisnil 25 mg (n = 11) mean ± SD	Asoprisnil 10 mg *vs.* placebo	Asoprisnil 25 mg *vs.* placebo
Baseline	213.0 ± 128.0	156.7 ± 103.8	217.9 ± 115.4	NA	NA
Final month	225.6 ± 232.7	2.4 ± 4.9	2.5 ± 8.1	NA	NA
Change from baseline to final month	12.6 ± 150.6	−154.3 ± 105.2	−215.4 ± 114.1	0.001[b]	<0.001[b]

Mean changes of MP scores (in milliliters) from baseline to final month in the three treatment groups (placebo, 10 mg asoprisnil, and 25 mg asoprisnil). NA, Not applicable.

[a] From ANCOVA model for change from baseline to final month with fixed effect of treatment and baseline score as a covariate.

[b] Statistical significance at 0.05 level using Hochberg's multiple-comparison procedure.

Ovarian activity

Urinary PdG levels (Table 4) were used to calculate luteal activity for three different time periods during treatment. In the 25-mg asoprisnil group, 70–80% of patients showed NELA during wk 9–12 of treatment compared with up to 20% in the placebo group. Dose-dependent suppression of luteal activity was apparent during wk 1–4 of treatment with 10–20% of patients showing NELA in the placebo group compared with 33% in the 10-mg asoprisnil and 80–90% in the 25-mg asoprisnil group.

Follicular activity indicated by urinary E_1G levels (Table 4) was partially or totally suppressed in 22% of patients on placebo compared with 33% on 10 mg asoprisnil and 60% on 25 mg asoprisnil. Continued follicular activity was seen in 78% of patients in the placebo group *vs.* 67 and 40% in the 10- and 25-mg asoprisnil groups, respectively. These results suggest a dose-dependent suppressive effect of asoprisnil on follicular activity.

Safety parameters

No asoprisnil-treated patient had a serious AE. No AEs led to discontinuation of study drug. The most common AEs reported by at least four patients in any group during treatment were headache, nasopharyngitis, nausea, back pain, perioperative complications, and abdominal pain. AEs exhibited no drug-related or dose-dependent pattern. There were no clinically meaningful mean changes from baseline in hematology, chemistry, and urinalysis laboratory values.

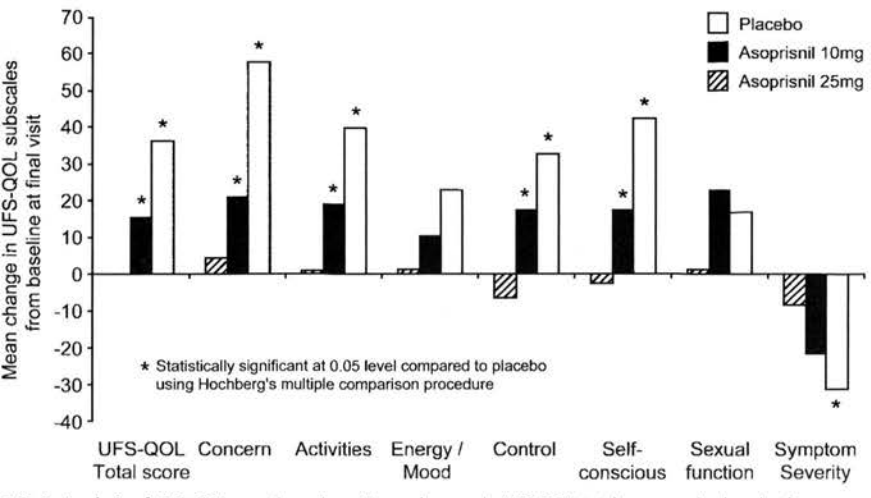

FIG. 2. Analysis of UFS-QOL questionnaires. Mean changes in UFS-QOL total score and subscales (concern, activities, energy/mood, control, self-consciousness, sexual function, and symptom severity) from baseline to final visit in the three treatment groups (placebo, 10 mg asoprisnil, and 25 mg asoprisnil) are shown. The significance of the difference of change from baseline between placebo and asoprisnil groups was determined using Hochberg's multiple-comparison procedure at 0.05 level. For symptom severity, a lower score corresponds to a lower severity; for other scales, a higher score indicates a better quality of life.

Discussion

The primary outcome of this study was evaluation of the effects of 3 months treatment with asoprisnil on uterine artery blood flow. Furthermore, effects of asoprisnil on uterine bleeding and quality of life measures were assessed in patients scheduled for hysterectomy due to symptomatic leiomyomata.

Asoprisnil treatment was associated with a moderately decreased uterine artery blood flow. There was a rapid reduction in uterine bleeding, evidenced by MP scores, and an improvement in quality of life measures.

Previous clinical studies suggested uterine artery blood flow to be important for leiomyoma growth (25). Pharmacological agents such as GnRH analogs (26) and danazol (27), which reduce leiomyoma volume, have been shown to reduce uterine artery blood flow. Furthermore, the progesterone antagonist mifepristone has been demonstrated to decrease uterine artery blood flow and reduce the size of the leiomyomatous uterus (10).

The effect of asoprisnil on uterine artery blood flow was assessed in this study to investigate a possible mechanism of action in patients with uterine leiomyomata. RI and PI were measured in uterine arteries to show a statistically significant effect on PI in both groups treated with asoprisnil and a trend toward increased RI compared with placebo. These findings suggest a moderate inhibitory effect of asoprisnil on uterine artery blood flow.

Asoprisnil has previously been shown to reduce leiomyoma volumes (15) with direct

TABLE 4. Urinary E_1G and PdG levels

	Treatment group		
	Placebo (n = 10) Mean ± SD	Asoprisnil 10 mg (n = 12) Mean ± SD	Asoprisnil 25 mg (n = 11) Mean ± SD
E_1G (μmol/mol)			
Screening	11.5 ± 4.0	18.0 ± 7.2	15.0 ± 5.5
wk 1–4	12.0 ± 4.9	16.2 ± 4.5	11.6 ± 3.3
wk 5–8	13.3 ± 3.4	17.5 ± 6.6	12.5 ± 5.4
wk 9–12	12.0 ± 4.0	21.0 ± 4.4	10.7 ± 2.0
PdG (mmol/mol)			
Screening	0.42 ± 0.15	0.37 ± 0.15	0.39 ± 0.22
wk 1–4	0.39 ± 0.17	0.45 ± 0.26	0.17 ± 0.11
wk 5–8	0.50 ± 0.25	0.45 ± 0.33	0.23 ± 0.19
wk 9–12	0.45 ± 0.19	0.46 ± 0.27	0.15 ± 0.10

Mean levels of urinary E_1G and PdG collected twice weekly over 4-wk intervals during the screening cycle and during treatment with placebo, 10 mg asoprisnil, 25 mg or asoprisnil. E_1G and PdG levels were measured using ELISA and hormone concentrations corrected for creatinine excretion. levels are expressed as ratios of the creatinine concentration (E_1G in micromoles per mole and PdG in millimoles per mole).

and indirect mechanisms likely to be involved. With the recognition that progesterone stimulates fibroid development and growth (6, 7), antiproliferative properties would be expected in a compound with partial progesterone antagonist effects. There is growing evidence from *in vitro* studies that asoprisnil suppresses proliferation and induces apoptosis in cultured leiomyoma cells while failing to show a similar effect on myometrial cells (28, 29). Evidence to date suggests that asoprisnil has selective antiproliferative effects on leiomyoma cells via down-regulation of growth factors and their receptors and induction of apoptosis (29) mediated by the PR. This is in contrast to the mode of action of GnRH analogs, which down-regulate ovarian estrogen and progesterone secretion via the pituitary gland to achieve a reduction in total uterine volume (30), whereas asoprisnil specifically decreases leiomyoma size. The present and previous studies have shown the antiproliferative effects of asoprisnil to occur in the presence of circulating follicular-phase estrogen concentrations (15).

Asoprisnil induces a constellation of endometrial morphological changes, which have been described as a nonphysiological secretory effect. In particular, administration of asoprisnil is associated with profound vascular changes with increased numbers of thick-walled stromal arterioles specific to the endometrium (16). These changes are associated with low levels of mitotic activity in endometrial glands and stroma, and no adverse endometrial findings such as endometrial hyperplasia or atypia have been demonstrated.

A further novel feature of this study is measurement of ovarian activity by assessing urinary PdG and E_1G twice weekly throughout the treatment period. There was an apparent dose-dependent suppression of luteal activity in asoprisnil-treated patients. Most patients experienced continued or only partially suppressed follicular activity on treatment with asoprisnil.

It should be stressed that luteinization in this study was defined based on urinary PdG concentrations typical for the normal luteal phase. Hence, luteal phase PdG may be indicative of either ovulation or a luteinized unruptured follicle. Serial ultrasound examinations of the dominant follicle and more frequent measurement of ovarian and pituitary hormones would be needed to determine the effects of asoprisnil on ovulation.

Previous studies have consistently reported asoprisnil to exert its clinical effects including suppression of menstruation in the presence of follicular-phase estrogen concentrations (15, 31). The risk of hypoestrogenism is the main limiting factor for the long-term use of GnRH analogs (32), which are currently often the only option for symptom control in patients with uterine fibroids seeking to avoid surgery.

The clinical effects of asoprisnil administered for 12 wk during this double-blind, placebo-controlled study are consistent with previous reports (15). A profound effect on menstrual bleeding was clearly demonstrated accompanied by a reduction in the severity of fibroid-related symptoms. Asoprisnil has previously been shown to dramatically reduce menstrual bleeding in women with (15) and without (31) fibroids. In this study, the effect of asoprisnil on endometrial bleeding was quantified using the MP. Consistent use of standardized sanitary products and provision of visual analogs on the pictogram allowed for quantification of menstrual blood loss, as previously described (20). Significant reductions were already apparent after the first month, highlighting a rapid effect of asoprisnil on uterine bleeding. Number of days with bleeding also markedly decreased in asoprisnil groups in a dose-related manner. Similarly, there was a dose response in the percentage of patients experiencing suppression of uterine bleeding. Treatment with 25 mg asoprisnil achieved suppression of uterine bleeding in 91% of patients, some of whom presented with MP scores of over 200 ml (definition of HMB is blood loss over 80 ml) (33). HMB is commonly difficult to manage in the presence of fibroids and frequent indication for hysterectomy. In this and previous studies (14, 15), asoprisnil has been shown to control uterine bleeding independent of size and location of uterine fibroids.

The mechanism of suppression of menstrual bleeding during asoprisnil treatment is not understood. Asoprisnil has previously been shown to reversibly suppress menstruation at doses of 10 mg/d or higher in women with regular menses. This effect was irrespective of the impact on luteal-phase progesterone concentrations indicative of luteinization (31). The results of the present study are consistent with these findings. Collectively, these observations strongly suggest that asoprisnil suppresses menstrual bleeding primarily via an endometrial effect. Asoprisnil induces

unique morphological changes in endometrial arterioles and stroma (16), and these changes are likely to contribute to the suppression of menstrual bleeding.

Asoprisnil-treated patients demonstrated statistically significantly greater improvements than placebo patients in most of the disease-specific UFS-QOL domains. Responses to the UFS-QOL questionnaire were grouped into subscales, as previously described (21). The mean change from baseline to the final visit indicated improvement in both asoprisnil groups compared with placebo on all subscales. These quality of life measures indicate a significant impact of asoprisnil on patients' perception of the severity of their symptoms and their quality of life. Every patient in this study had experienced symptoms significant enough to consent to major surgery for benign disease. In this study, treatment with asoprisnil was well tolerated. There were no premature terminations, and all patients completed the study with good compliance.

In conclusion, we have made the novel observations that asoprisnil reduces uterine artery blood flow while substantially decreasing menstrual blood loss and improving quality of life measures in patients with symptomatic uterine leiomyomata scheduled for hysterectomy. A moderate reduction in uterine artery blood flow was demonstrated by change in resistance and pulsatility indices. This effect may contribute to leiomyoma volume reduction, even though it is unlikely to be the primary mechanism. Decreased blood loss was evidenced by MP evaluation and improvement of quality of life by responses to the UFS-QOL. All these effects were observed in the presence of continued or only partially suppressed ovarian follicular activity in the majority of patients. The 10- and 25-mg doses of asoprisnil were safe and effective when administered daily for a 12-wk period. Further studies are needed to determine safety and efficacy profiles of asoprisnil when administered beyond 12 wk.

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The effects of the selective progesterone receptor modulator asoprisnil on the morphology of uterine tissues after 3 months treatment in patients with symptomatic uterine leiomyomata

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ACKGROUND: Asoprisnil is a selective progesterone receptor modulator with mixed progesterone agonist/ ntagonist activity which controls uterine bleeding via an endometrial effect. This study examined full-thickness endo- etrial, leiomyoma and myometrial morphology in hysterectomy specimens from patients with uterine leiomyomata, fter treatment with asoprisnil for 3 months. METHODS: In this double-blind, randomized, placebo-controlled study, 3 subjects with uterine leiomyomata were randomized to receive asoprisnil 10, 25 mg or placebo for an average of 95 ays prior to hysterectomy. Samples of endometrium, myometrium and leiomyoma tissue were subjected to systema- c morphological assessment with quantification of mitotic activity. RESULTS: In patients treated with 10 or 25 mg soprisnil, a unique pattern called 'non-physiologic secretory effect' was evident in endometrium, recognizable rough partially developed secretory glandular appearances and stromal changes. Endometrial thickness was ecreased, and there were low levels of mitotic activity in endometrial glands and stroma. Unusual thick-walled mus- ular arterioles and prominent aggregations of thin-walled vessels were present in endometrial stroma, but not in yometrium or non-endometrial vascular beds. Mitotic activity was decreased in leiomyomata. CONCLUSIONS: soprisnil induces unique morphological changes and is associated with low levels of glandular and stromal prolifer- ion in endometrium, and in leiomyomata. These changes are likely to contribute to the amenorrhoea experienced fter exposure to the medication.

eywords: asoprisnil/cell-proliferation/endometrium/histopathology/progesterone receptor

ntroduction

soprisnil is a member of a developing class of selective pro- esterone receptor modulators (SPRMs) being studied in omen with symptomatic uterine leiomyomata. SPRMs are rogesterone receptor (PR) ligands that exert clinically relevant ssue-selective progesterone agonist, antagonist or partial or ixed agonist/antagonist effects on various progesterone rget tissues in an *in vivo* situation depending on the biological ction studied (Chwalisz *et al.*, 2005b). Asoprisnil, a steroidal 1β-benzaldoxime-substituted SPRM, exhibits mixed pro- esterone agonist/antagonist activities in animals (DeManno *al.*, 2003; Schubert *et al.*, 2005), and high degree of erine selectivity in women (Chwalisz *et al.*, 2005a). In contrast to pure progesterone antagonists that inhibit ovulation and do not show tissue-selective effects, asoprisnil suppresses uterine bleeding in the presence of follicular phase estrogen concentrations irrespective of the effects on ovulation. It controls uterine bleeding in a dose-dependent manner by an unknown mechanism and appears to target endometrium specifically (Chwalisz *et al.*, 2005a). In addition, asoprisnil exerts endometrial antiproliferative effects in non-human primates (Chwalisz *et al.*, 2005b) and reduces leiomyoma volume in humans (Chwalisz *et al.*, 2003). These effects provide the rationale for its use in the treatment of uterine leiomyomata, in which abnormal uterine bleeding is a common symptom.

Uterine leiomyomata (fibroids or myomas) are common benign smooth muscle neoplasms originating in myometrium. They occur in at least 50% of women of reproductive age, and although many are asymptomatic, they commonly lead to troublesome symptoms of heavy menstrual bleeding, as well as pressure symptoms and infertility. Symptoms attributable to leiomyomata are the primary indication for 200 000–300 000 hysterectomies performed each year in the USA among premenopausal women (Schwartz, 2001). At present, there is no entirely satisfactory medical treatment for management of women with symptomatic leiomyomata.

The present randomized double-blind placebo-controlled study was therefore designed to allow detailed histological assessment of full-thickness endometrium, myometrium and leiomyomata in samples from hysterectomy specimens of patients with symptomatic leiomyomata who had been administered 10 or 25 mg asoprisnil daily for 12 weeks prior to surgery. These doses were selected as previous studies (Chwalisz *et al.*, 2005a, b) have shown dose-dependent reversible suppression of menstruation at doses $\leq$10 mg, and dose-dependent reduction of leiomyomata and uterine volume.

The focus of this study was on the endometrial effects. The endometrium seems to be the most sensitive target for SPRMs, since the effects of asoprisnil on uterine bleeding and endometrial morphology were evident at doses that did not markedly inhibit luteinization indicative of ovulation and ovarian estrogen secretion. In addition, compounds with partial progesterone antagonist effects have the potential to induce 'unopposed' estrogen effects on the endometrium and create, therefore, concerns about endometrial safety. Finally, early studies with asoprisnil revealed that this compound induces unique changes in the endometrium, characterized as 'non-physiologic secretory effects' that have not been described with any known pharmacological agent before (Chwalisz *et al.*, 2005a). It was clear in this and other unpublished studies that hitherto undescribed changes were present in the endometria of asoprisnil-treated patients, and conventional assessment using the criteria of Noyes *et al.* (1950) was not appropriate. The evaluation of full thickness, highly oriented endometrial specimens obtained during hysterectomy provided a unique opportunity to study the effects of asoprisnil in all layers of the endometrium, including the basalis, which is not accessible by Pipelle endometrial biopsies.

Materials and Methods

Women studied

The study group was composed of premenopausal women volunteers from four centres (Edinburgh, Southampton, Glasgow and Liverpool) in good general health with a menstrual cycle between 17 and 42 days, and symptoms related to overall fibroid size, pressure and/or heavy uterine bleeding (per protocol), who were scheduled for hysterectomy. Each patient had at least one intramural non-pedunculated, submucosal or subserosal fibroid with a diameter of at least 2 cm or multiple small fibroids with uterine volume $\geq$200 cm^3 on ultrasonography. Other inclusion criteria included age over 18 years; negative pregnancy test; a washout period of 2–12 months for hormonal therapies; serum FSH of $<$30 mIU ml^{-1} at commencement; agreement to use double barrier method of contraception (condom/diaphragm/sponge plus spermicide) throughout the study until hysterectomy, unless surgically sterile by bilateral tubal ligation or vasectomy of partner and normal Papanicolaou (PAP) test. Subjects were not permitted to enter the study without a normal endometrial biopsy report based on an adequate specimen taken within 3 months of commencement. Screening data were collected in the clinic by study nurses or gynaecologists. All subjects voluntarily signed a full informed consent form. The study was performed according to the ethical principles of the Declaration of Helsinki (1989 revision) and the protocol was approved by the Institutional Review Board (Multicentre Research Ethics Committee).

Study design

This was a Phase 2 multicentre randomized double-blind placebo-controlled study of asoprisnil administered for 12 weeks. Dose selection was based upon data from Phase I and Phase II studies (publications in preparation) in which asoprisnil at doses of 5, 10, and 25 mg has been shown to be safe in subjects treated for 12 weeks. Asoprisnil 10 and 25 mg doses and the 12-week duration of treatment were chosen because they were effective for treatment of excessive bleeding, in reducing leiomyoma and uterine volumes and in decreasing mass effect symptoms, such as pelvic pressure and bloating, over a 3-month treatment period.

At baseline, screening procedures (performed within 45 days of study commencement) included pelvic and breast examination, complete physical examination, PAP test (ThinPrep® Pap Test, Cytyc Corp), ECG, transvaginal ultrasound, endometrial biopsy (Unimar Pipelle® Endometrial Suction Curette, Medscand), contraception counselling and clinical laboratory investigations: chemistry, haematology, urinalysis, endocrine panel, lipid profile, coagulation screen and serum and urine pregnancy tests. After successful enrollment based on inclusion and exclusion criteria, women were sequentially assigned subject numbers in ascending numerical order that encoded the assignment of the woman, via a randomization schedule, to one of the three treatment arms of the study. Subjects were randomized to one of three parallel dose groups in a 1:1:1 ratio to receive daily doses of asoprisnil 10, 25 mg or placebo. Subjects and all study personnel were blinded to treatment groups. Asoprisnil or placebo tablets were supplied in blister cards of identical appearance, supplied to the site packaged in sealed kits. Drug was self-administered as an oral dose taken once daily. Treatment was initiated no later than the fifth day of the subject's menstrual cycle, and continued for 12 weeks, when subjects were to undergo hysterectomy within 24 h of the final dose of drug. The subjects returned all used and unused study medication blister cards at 8-week and final visits so that verification of medication compliance could be monitored. The effects of asoprisnil on clinical and safety parameters will be reported separately.

Hysterectomy and processing of uterine samples

After removal of the uterus, the unfixed specimen was placed on ice immediately and taken without delay to the local pathology laboratory, where the study pathologist or deputy was on hand to open and sample the specimen. The specimen was oriented and a probe inserted through the external os of the cervix to define the position of the cavity. The uterus was opened using a long-bladed knife along the plane of the probe. The opened specimen was then placed in an adequate volume of 10% buffered formaldehyde and allowed to fix overnight. For large specimens, parallel parasagittal slices 2 cm in thickness were made to permit adequate fixation overnight. The following day, the pathologist sampled the specimen for routine diagnostic reporting, and took additional blocks for study assessment. The additional blocks consisted of three blocks from the uterine fundus,

id-corpus and isthmus, respectively, ideally to include full thickness om endometrium, myometrium and serosal surface, but in any case cluding full thickness of endometrium with underlying myome- ium. Endometrial samples were taken from areas away from leio- yomata wherever possible. Leiomyomata were separately sampled. ll specimens were processed by routine methods to paraffin wax, id 3 μm haematoxylin–eosin sections were prepared by microtomy id mounted on glass slides. Histological sections of endometrium ere considered unsuitable for assessment if a leiomyoma was esent within 10 mm of the endometrium.

istological assessment of endometrium

uring Phase I studies with asoprisnil, it became clear that SPRMs cluding asoprisnil induce unique changes in the endometrium that innot be assessed by currently used criteria of endometrial dating e. the Noyes criteria) (Noyes *et al.*, 1950), because of differential fects on glands and stroma. As a result, TAP Pharmaceutical Pro- ucts Inc., Lake Forest, IL, Diagnostic Cytology Laboratories, India- apolis, IN, USA, and a group of expert gynaecological pathologists veloped a list of diagnostic criteria that allows for classification of anges induced by SPRMs ('Dictionary of Endometrial Biopsy Diag- oses for Clinical Trials with SPRMs'—data on file). This system sup- ements the conventional descriptive criteria of the normal menstrual cle as described by Noyes, and in Blaustein's Pathology of the Female Genital Tract (Mutter and Ferenczy, 2002), but includes additional categories that are considered characteristic of the effects of SPRMs.

Haematoxylin–eosin stained sections of full-thickness endometrium and underlying myometrium from fundus, mid-corpus and isthmus and samples of fibroids were evaluated by a gynaecological pathologist blinded to treatment groups. The maximum single endometrial thickness on each section was measured using an eyepiece micrometer. Overall endometrial appearances were classified according to the classification system described earlier. Individual histological characteristics of endometrial glands, stroma and vessels were analysed by assigning a descriptive subcategory, as shown in Tables 1 and 2. Myometrium was assessed for any histological changes or abnormalities, and for the presence or absence of adenomyosis. Leiomyomata were assessed for histological type (usual, cellular, epithelioid, symplastic, myxoid, leiomyoma with heterologous elements, other) and for degenerative effects (no degenerative changes, hyaline degeneration, cystic degeneration, myxoid or mucoid degeneration, coagulative necrosis, calcification, fatty degeneration).

Non-endometrial tissues, when submitted with the hysterectomy specimens, were also examined histologically. These comprised samples of ovaries, Fallopian tubes and cervix. As these were the only non-uterine tissue samples available, it was of particular interest to assess the vascular beds to determine whether the changes in endometrial vessels were specific to that site, or could be seen in other tissues.

Assessment of mitotic activity

Glandular and stromal mitoses per unit area of endometrium were quantified separately by counting the number of unequivocal mitotic figures present per 10 high-power microscope fields of endometrium (Olympus BX51, ×40 objective, field diameter 0.55 mm). Counting was commenced in the area identified by prior microscopic assessment

ıble 1. Morphological categories of endometrial glandular histology

orphology	Category
ıriants of normal cycle	Simple tubules, minimum undulation
	Simple tubules, undulation as seen in normal proliferative phase
	Tubules, undulation as seen in early secretory phase
	Glands with morphology of mid or late secretory phase
	Mixed (less than 80% show one pattern)
'stic dilatation	No cystic dilation
	<10% of glands
	10–25% of glands
	>25% of glands
lding patterns[a]	No abnormal folding
	1–2 glands
	3–6 glands
	>6 glands
and-stroma ratio	1:>5
	Between 1:5 and 1:1
	>1:1 and <3:1
	≥3:1
ll height	'Flattened' (cell height < cell width)
	'Cuboidal' (cell height = cell width)
	'Columnar' (cell height > cell width)
cretory effects	No cytoplasmic vacuolation
	Sub and/or supranuclear vacuolation
	Apical secretion, with or without cytoplasmic vacuolation
	Intraluminal secretion
ıclear stratification	None
	<10%
	10–50%
	>50%
ıclear atypia	Absent
	Present in 1–2 glands
	Present in >2 glands, up to 50%
	Present in >50% of glands

bnormal folding patterns as seen in disordered proliferative pattern, pressed as number of abnormally folded glands per slide; no focus >2 mm ameter.

Table 2. Morphological categories of endometrial stromal histology

Morphology	Category
Cellularity[a]	Compact atrophic[b]
	Variable oedema, atrophic
	Oedematous atrophic
	Compact non-atrophic
	Variable oedema, non-atrophic
	Oedematous, non-atrophic
Decidual change	None
	Around vessels only
	Around vessels, extending into stroma (non-confluent)
	Confluent decidual change
Vessels: thin walled	No dilatation
	<50% show dilatation
	≥50% show dilatation
Vessels: muscularization	No mural muscularisation
	<50% show normal mural muscularisation
	≥50% show normal mural muscularisation
	Abnormally muscularised[c] arterial vessels present
Vessels: aggregations[d]	None
	1–2
	>2

[a]Non-decidualized stroma only.
[b]Atrophic defined as stromal cells have nucleus/cytoplasm (NC) ratio ≥1.
[c]Abnormal arterial vessels similar to those seen in stroma of endometrial polyps.
[d]Number of vessel aggregates ('tangles') per slide.

to show the greatest density of mitoses, and a minimum of 10 non-overlapping fields randomly selected for counting thereafter. The same method was used to assess mitotic activity in leiomyomata, but uninvolved myometrial tissue was not assessed in this way as it shows no mitotic activity.

Data analysis and statistical methods

For the overall diagnostic category of endometrium as well as categorical assessments of morphological features in glands and stroma, number and percentage of patients in each category were summarized for each treatment group and each of the three locations (uterine fundus, mid-corpus and isthmus), with no statistical inference performed. For endometrial thickness measured by eyepiece micrometer, mean and standard deviation were calculated for each treatment group and each of the three locations. Although not prespecified, the thickness was also compared, for each location separately, between the placebo group and the combined asoprisnil group, using *t*-tests. The multiplicity resulting from the three locations was adjusted for using Hochberg's multiple comparison procedure.

No sample size calculation was performed for this mechanism of action study. Although a power analysis was carried out for resistance index (primary efficacy endpoint), and a uterine blood flow parameter assessed with colour Doppler imaging in this study, none was performed for morphological characteristics as no statistical inference was to be performed on these characteristics.

Results

Demographic information

There were 10, 12 and 11 subjects, respectively, who received placebo, asoprisnil 10 and 25 mg. Twenty-nine subjects were Caucasian and four were black. Placebo and asoprisnil groups (10 and 25 mg) were well matched with respect to age (mean 41.8, 45.1 and 44.6 years, respectively), weight (mean 73.4, 73.8 and 75.9 kg) and height (165.3, 164.3 and 165.6 cm). The treatment period for this study was between July 2003 and March 2005. Drug compliance was satisfactory in all groups. On average, the subjects took the study drug for 95 days. All hysterectomies were performed within 2 days of the last dose of study drug.

Histological evaluation of endometrium

Baseline endometrial biopsies taken prior to commencement of medication all showed normal histological appearances, classified as inactive (10%), proliferative (28%), menstrual (7%) or secretory—cycling/physiologic (55%).

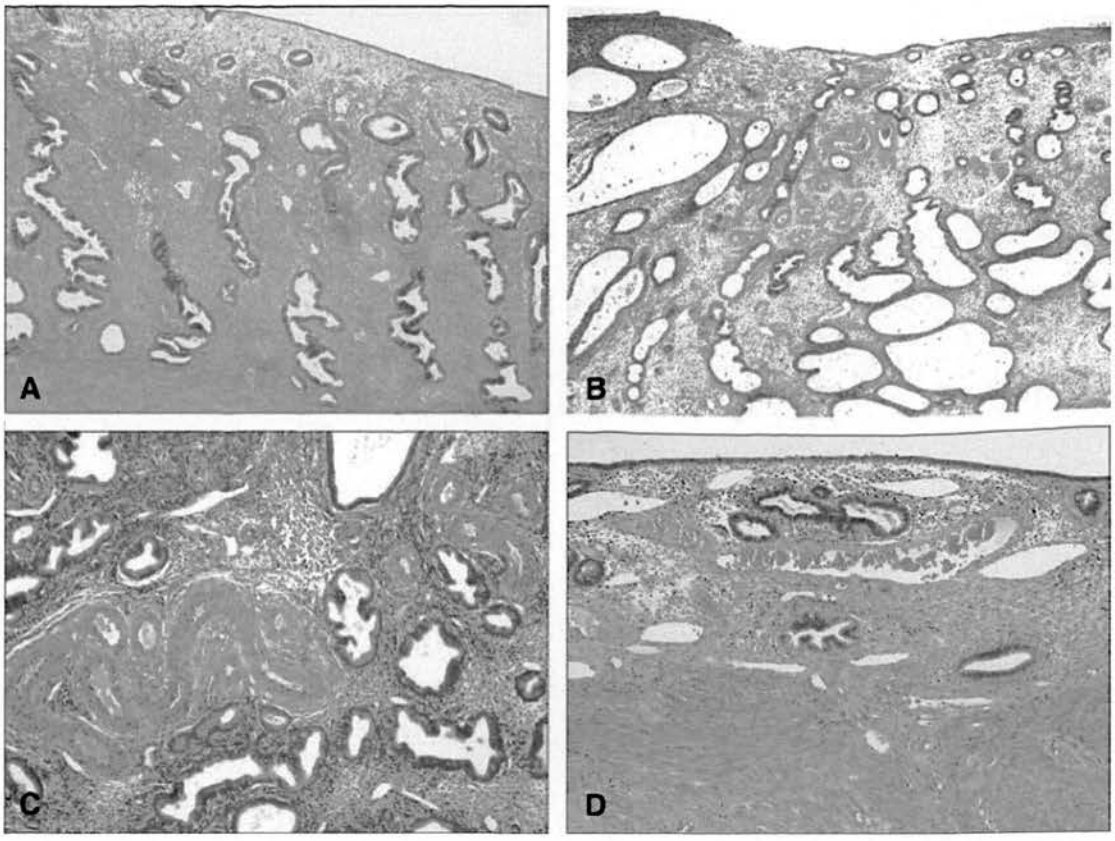
In 29 subjects, endometrial samples from fundus, mid-corpus and isthmus were taken, and were available for histological assessment. Table 3 shows the overall diagnostic category assigned to endometrium from three locations (fundus, mid-corpus and isthmus) in specimens from subjects taking placebo, 10 or 25 mg asoprisnil. In fundus, mid-corpus and isthmus (this order will be used throughout the text), respectively, normal secretory appearances (cycling/physiologic) were seen in five of seven, five of seven and five of seven samples from patients taking placebo, whereas specimens from patients taking 10 or 25 mg asoprisnil showed normal secretory appearances in 1 of 10, 2 of 10 and 0 of 10 and in 1 of 11, 1 of 11 and 0 of 10, respectively. In contrast, a non-physiologic secretory effect was found in 7 of 10, 5 of 10 and 5 of 10 samples from patients taking 10 mg asoprisnil, and 8 of 11, 8 of 11 and 5 of 10 samples from patients taking 25 mg. The diagnosis of non-physiologic secretory effect was assigned in one of seven, zero of seven and one of seven samples from patients receiving placebo.

Overall description of endometrial morphology associated with asoprisnil

Endometrium of patients treated with asoprisnil showed a combination of morphological features affecting glands, stroma and vessels. Although none of these features was on its own specific for asoprisnil, the occurrence of several features together allowed designation of the endometrium as showing the 'non-physiologic secretory effect'. In this pattern, the endometrial glands showed some features associated with secretory differentiation, often with serpentine or tortuous profiles, composed of columnar epithelial cells with infrequent or absent mitotic activity (see Fig. 1A as an example). However, glands rarely showed any evidence of active secretion, as cytoplasmic vacuolation was absent in most cases. There was a tendency for cystic dilatation of glands, but no abnormal folding patterns or increase in gland-stroma ratio above normal (Fig. 1B). The stroma showed a tendency for increased cellularity without evidence of decidual change. One of the most consistent changes with asoprisnil was the effect on vessels, of which there were two characteristic appearances. First, thick-walled muscularized vessels, similar to those seen in the stroma of the common benign endometrial polyp, were commonly found in the stroma of asoprisnil-treated patients (Fig. 1C), but were not identified in any subjects receiving placebo. Secondly, aggregates of thin-walled vessels were seen at all levels of

Table 3. Overall diagnostic category assigned to endometrium in specimens from subjects taking placebo, 10 or 25 mg asoprisnil

Site in uterus	Placebo, *n* (%)			Asoprisnil 10 mg, *n* (%)			Asoprisnil 25 mg, *n* (%)		
	Fundus *n* = 7	Corpus *n* = 7	Isthmus *n* = 7	Fundus *n* = 10	Corpus n = 10	Isthmus *n* = 10	Fundus *n* = 11	Corpus *n* = 11	Isthmus *n* = 10
Diagnostic category									
Inactive	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	1 (10)	1 (9)	1 (9)	3 (30)
Normal proliferative	1 (14)	1 (14)	1 (14)	2 (20)	2 (20)	3 (30)	1 (9)	0 (0)	1 (10)
Normal secretory	5 (71)	5 (71)	5 (71)	1 (10)	2 (20)	0 (0)	1 (9)	1 (9)	0 (0)
Non-physiologic secretory	1 (14)	1 (14)	0 (0)	7 (70)	5 (50)	5 (50)	8 (73)	8 (73)	5 (50)
Unsatisfactory	0 (0)	0 (0)	1 (14)	0 (0)	0 (0)	1 (10)	0 (0)	1 (9)	1 (10)



gure 1. (**A**) Glands show a sinuous or serpentine profile, similar to the architecture of glands seen in the mid-secretory phase of the menstrual cle. (**B**) Glands frequently show cystic dilatation. Note the thick-walled vessel in endometrial stroma. (**C**) Thick-walled muscularized vessel in dometrial stroma. (**D**). Dilated thin-walled vessels in thin endometrium with widely dispersed sinuous glands in loose non-decidualized stroma.

e endometrium, from within stratum basalis to just beneath rface epithelium (Fig. 1D). These were not specific to aso- snil, as they were occasionally observed in placebo-treated tients, but they were much more common among asoprisnil- ated patients. In asoprisnil-treated patients, endometrium s commonly thin (Fig. 1D) and frequently it was not possible distinguish stratum basalis from the stratum functionalis. e morphological features associated with asoprisnil were nilar in the thin endometrium of both basalis and nctionalis.

dividual features of endometrial glands

ith asoprisnil, cytoplasmic secretory vacuolation was rela- ely infrequent (up to 30%) in patients taking 10 mg, and sent from glands in all locations in all patients taking mg. With asoprisnil, gland architecture varied from simple bules with minimal undulation to a tortuous appearance embling that of the mid- or late secretory phase. Simple bular gland architecture with minimal undulation was more mmonly seen in endometrium from the isthmus in patients king 25 mg (40%) than in fundus or mid-corpus (9%). Aso- snil 25 mg led to some degree of cystic gland dilatation in uterine locations but especially in the isthmus (80% com- red to 29% with placebo). No differences in the frequency other glandular architectural abnormalities, such as the normal folding patterns seen in the disordered proliferative pattern (Mutter and Ferenczy, 2002) were identified between asoprisnil-treated patients and controls. The predominant epithelial cell type seen in glands in all locations was comparable between asoprisnil and placebo, consisting of tall columnar cells showing a degree of nuclear stratification that did not vary significantly between treatment groups. Epithelial cell nuclei showed no cytological atypia in any patient. Although a systematic assessment was not performed, histologically there was no apparent difference in numbers of apoptotic glandular cells between asoprisnil-treated patients and controls.

Mitotic activity in endometrial glands and stroma

Mitotic activity for endometrial glands is shown in Table 4 and for stroma in Table 5. As five out of seven, five out of seven and five out of seven samples from placebo-treated patients showed normal secretory endometrial appearances in which mitoses are very infrequent or absent, and only one out of seven, one out of seven and one out of seven specimens showed normal proliferative appearances (Table 3), mitotic activity cannot properly be compared between asoprisnil-treated patients and the placebo group. However, it is notable that in the group of patients treated with 25 mg asoprisnil, no mitotic activity in endometrial glands was identified in any specimen except for one sample of fundic endometrium in one patient. At least 70% of specimens from patients taking

Table 4. Mitotic activity in endometrial glands

Site in uterus	Placebo, *n* (%)			Asoprisnil 10 mg, *n* (%)			Asoprisnil 25 mg, *n* (%)		
	Fundus $n=7$	Corpus $n=7$	Isthmus $n=7$	Fundus $n=10$	Corpus $n=10$	Isthmus $n=10$	Fundus $n=11$	Corpus $n=11$	Isthmus $n=9$
No. of gland mitoses per 10 HPF[a]									
0	5 (71)	5 (71)	4 (57)	5 (50)	5 (50)	5 (50)	9 (82)	10 (91)	9 (100)
1	0 (0)	0 (0)	0 (0)	3 (30)	2 (20)	2 (20)	1 (9)	0 (0)	0 (0)
2–5	1 (14)	0 (0)	1 (14)	1 (10)	2 (20)	0 (0)	0 (0)	0 (0)	0 (0)
>5	1 (14)	2 (29)	1 (14)	1 (10)	1 (10)	1 (10)	0 (0)	0 (0)	0 (0)
Unsatisfactory	0 (0)	0 (0)	1 (14)	0 (0)	0 (0)	2 (20)	1 (9)	1 (9)	0 (0)

[a]High-power field (HPF) (Olympus BX51, 40× objective, field diameter 0.55 mm).

10 mg asoprisnil showed one mitosis or less per 10 high-power fields (HPF) in endometrial glands. In endometrial stroma, patients who received 25 mg asoprisnil showed absence of mitotic activity in 8 out of 11, 9 out of 11 and 9 out of 9 assessable slides, and with 10 mg, 1 mitosis or less per 10 HPF was seen in 7 out of 10, 10 out of 10 and 8 out of 10 assessable slides.

Stromal changes

Stromal decidual change was seen around vessels in a specimen from fundus only in one patient taking 25 mg asoprisnil, but in the remaining assessable samples from patients taking either 10 or 25 mg asoprisnil there was no decidual change identified. In contrast, decidual change was present in endometrial stroma in three of seven, three of seven and three of seven samples from patients taking placebo. With asoprisnil, there was an increased frequency of stromal compactness in all uterine locations, compared with placebo. Stroma was assessed as compact in zero of seven, zero of seven and one of seven placebo samples, compared to 6 of 10, 3 of 10 and 3 of 10 samples from patients taking 10 mg asoprisnil and 6 of 11, 5 of 11 and 7 of 10 on 25 mg.

Endometrial thickness

Table 6 shows the mean single layer thickness of the endometrium, as measured by eyepiece micrometer on histological sections, for specimens from patients taking placebo, 10 or 25 mg asoprisnil. There was no statistically significant difference in endometrial thickness between placebo and combined asoprisnil groups, although there was a trend for decreased thickness with asoprisnil treatment.

Vessels

Aggregates or leashes of thin-walled vessels were seen with increased frequency in endometrial stroma of asoprisnil-treated patients compared with controls (Table 7). Samples from patients on placebo showed the presence of at least one aggregation of thin-walled vessels in 2 of 7, 1 of 7 and 0 of 7, whereas these were present in 3 of 10, 5 of 10 and 2 of 10 samples from patients on 10 mg asoprisnil and in 5 of 11, 5 of 11 and 4 of 10 on 25 mg.

Thick-walled vessels, similar to those seen in the stroma of endometrial polyps, were seen with increased frequency compared with controls in all uterine locations in patients treated with asoprisnil. Such vessels were not seen in any samples from placebo subjects, whereas they were found in 2 of 10, 3 of 10 and 1 of 10 samples from patients on 10 mg asoprisnil and in 5 of 11, 5 of 11 and 3 of 10 on 25 mg.

Although there are some similarities, the changes associated with asoprisnil differ from those associated with endometrial polyps. In the latter, thick-walled vessels occur in the stroma of a rounded, projecting polypoid mass in which gland crowding may be observed, and the stroma frequently has a collagenous appearance. After 12 weeks treatment with asoprisnil, gland crowding is not seen, the stroma does not appear prominently collagenized, and the endometrium remains flat and often thinned.

Non-endometrial vascular beds

There were 12 ovaries from eight patients, 15 Fallopian tubes from 10 patients and 10 cervices available for assessment of vascular beds in non-endometrial tissues. No specific histological abnormalities were observed.

Table 5. Mitotic activity in endometrial stroma

Site in uterus	Placebo, *n* (%)			Asoprisnil 10 mg, *n* (%)			Asoprisnil 25 mg, *n* (%)		
	Fundus $n=7$	Corpus $n=7$	Isthmus $n=7$	Fundus $n=10$	Corpus $n=10$	Isthmus $n=10$	Fundus $n=11$	Corpus n = 11	Isthmus $n=9$
No. of stromal mitoses per 10 HPF[a]									
0	3 (43)	2 (29)	3 (43)	6 (60)	7 (70)	6 (60)	8 (73)	9 (82)	9 (100)
1	2 (29)	1 (14)	0 (0)	1 (10)	3 (30)	2 (20)	1 (9)	0 (0)	0 (0)
2–5	0 (0)	4 (57)	3 (43)	3 (30)	0 (0)	0 (0)	1 (9)	1 (9)	0 (0)
>5	2 (29)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Unsatisfactory	1 (9)	0 (0)	1 (14)	0 (0)	0 (0)	2 (20)	1 (9)	1 (9)	0 (0)

[a]High-power field (Olympus BX51, 40× objective, field diameter 0.55 mm).

ıble 6. Mean endometrial thickness measured by eyepiece micrometer on histological sections from hysterectomy specimens

erine cation	Placebo			Asoprisnil 10 mg			Asoprisnil 25 mg		
	n	Mean	SD	*n*	Mean	SD	*n*	Mean	SD
ndus	7	3.60	1.988	10	2.62	1.307	11	2.79	1.718
id-corpus	7	4.06	2.018	10	2.14	0.977	11	2.22	1.098
hmus	6	3.05	1.907	9	2.26	1.840	10	1.27	0.625

= total no of cases. The apparent trend of decreasing endometrial thickness with asoprisnil at both doses is not statistically significant at 0.05 level.

ıble 7. Endometrial stromal vessels

te in uterus	Placebo, *n* (%)			Asoprisnil 10 mg, *n* (%)			Asoprisnil 25 mg, *n* (%)		
	Fundus $n=7$	Corpus $n=7$	Isthmus $n=7$	Fundus $n=10$	Corpus n = 10	Isthmus $n=10$	Fundus $n=11$	Corpus $n=11$	Isthmus $n=10$
essels									
in walled[a]	2 (29)	1 (14)	0 (0)	3 (30)	5 (50)	2 (20)	5 (45)	5 (45)	4 (40)
ick walled[b]	0 (0)	0 (0)	0 (0)	2 (20)	3 (30)	1 (10)	5 (45)	5 (45)	3 (30)

ggregates of thin-walled vessels present.
hick-walled muscularized vessels present.

yometrium

yometrium samples were obtained from all 33 patients and orphological assessments were performed. Adenomyosis as infrequent (present in one control subject, one taking) mg and four taking 25 mg asoprisnil). No further abnormal-es or changes were identified in these samples.

iomyomata

istological evaluation of the largest leiomyoma of each sterectomy specimen showed 29 of 30 to be of the usual stological type, and one to be of cellular type. Degenerative anges (hyaline or myxoid/mucoid changes, or coagulative crosis) were seen in 4 of 9 leiomyomata from the placebo oup, 8 of 12 in the 10 mg and 7 of 9 in the 25 mg asoprisnil oup. In the placebo group, mitotic activity was not entifiable in one of eight leiomyomata, and in the range -2 mitoses per 10 HPF in the remaining seven of eight sessable leiomyomata (Table 8). In the 10 mg asoprisnil oup, 8 of 12 showed no identifiable mitoses and 4 of 12 owed 1–2 mitoses per 10 HPF. In the 25 mg asoprisnil oup, six of nine showed no mitoses, two cases showed 1–2 itoses per 10 HPF and one case showed 3–5 mitoses per) HPF.

ıble 8. Mitotic activity in largest leiomyoma

o. of mitoses r 10 HPF[a]	Placebo (N=9), *n* (%)	Asoprisnil 10 mg (N=12), *n* (%)	Asoprisnil 25 mg (N=9), *n* (%)
	1 (11)	8 (67)	6 (67)
-2	7 (78)	4 (33)	2 (22)
-5	0 (0)	0 (0)	1 (11)
5	0 (0)	0 (0)	0 (0)
ısatisfactory	1 (11)	0 (0)	0 (0)

igh-power field (Olympus BX51, 40× objective, field diameter 0.55 mm).

Discussion

Asoprisnil has a variety of clinical effects that have so far not been described with any other pharmaceutical agent. It controls uterine bleeding in a dose-dependent manner without causing suppression of estradiol from follicular phase levels and irrespective of the effects on luteinization (Chwalisz *et al.*, 2005a). In patients with leiomyomata, it suppresses heavy uterine bleeding, reduces leiomyoma and uterine volumes and has beneficial effects on pressure-related symptoms (Chwalisz *et al.*, 2003). These distinctive clinical effects are associated with equally distinctive morphological effects on endometrium, and the existing compendium of pathological diagnoses does not include appropriate categories to describe the effects of asoprisnil on endometrium. Early clinical studies (Chwalisz *et al.*, 2005a) have examined the asoprisnil effect in endometrial biopsies taken by Pipelle aspiration after treatment for 28 days. The present study, however, has allowed for the first time, a detailed morphological investigation of samples of full thickness endometrium and underlying myometrium, mostly well oriented and undisrupted by the sampling procedure after a 3-month asoprisnil treatment. Most of the subjects recruited were suffering symptoms of heavy uterine bleeding associated with fibroids, and the endometrium cannot, therefore, be considered functionally normal. Although efforts were made to sample endometrium at least 10 mm away from the nearest fibroid, it is known that vascular changes occur in the endometrium of these patients, and results must be interpreted with caution. However, the baseline endometrial biopsies taken at screening were within normal limits histologically.

The majority of asoprisnil-treated subjects showed endometrial morphology that was consistently recognizable as showing a 'non-physiologic secretory effect', a category that was assigned in only one placebo-treated subject. It was

present to a similar extent in all three uterine locations sampled (fundus, mid-corpus and isthmus), and was seen in a higher proportion of subjects on 25 mg of asoprisnil. This appearance is unfamiliar to histopathologists, as it is not seen in normal cycling endometrium or in any described condition, and is believed to reflect the partial agonist/antagonist effect of asoprisnil described in animal studies (DeManno *et al.*, 2003). The histological features defining the effect are endometrial glands showing tortuosity similar to the architecture of the secretory phase, but with a rarity of secretory activity or cytoplasmic vacuolation. Glands frequently showed cystic dilatation, however, and although nuclear stratification was present, there was a paucity of mitotic activity. Appearances of cystic glandular dilatation are frequently associated in the minds of histopathologists with simple hyperplasia of endometrium, but the non-physiologic secretory effect differed in that glandular crowding was absent, cystic change was often focal involving one or two glands in a field of non-dilated glands and mitotic activity was very sparse or absent. Gland dilatation is a feature commonly seen in the inactive, non-secretory endometrium of the perimenopausal and post-menopausal woman, and is not considered indicative of glandular secretory activity. It may in fact represent glands in which the opening to the surface has become occluded, perhaps, as a consequence of the lack of secretory activity.

In this 3-month study, endometrial thickness was not increased in the asoprisnil group compared with placebo, and indeed the trend was for mean endometrial thickness to be less than that of placebo. With asoprisnil, the endometrial stroma showed increasing compactness without decidual change, but the most characteristic stromal effect involved vessels. Aggregations of thin-walled vessels were seen in endometrial stroma more frequently in asoprisnil-treated patients than in placebo-treated patients. Additionally, vessels with thickened muscularized walls were seen with greater frequency in asoprisnil patients than in controls.

The mechanism of amenorrhoea induced by asoprisnil is not known, but it is probable that the drug exerts a specific effect on endometrial vessels through action on the PR. This may involve perivascular cells that express PR, and are believed to be pivotal in control of menstruation (Kelly *et al.*, 2002), or other stroma-mediated events. The perivascular cells are intimately apposed to the endothelial cells of spiral arterioles, and are believed to respond to falling progesterone concentrations by initiating vasoconstriction–vasodilatation cycles with resulting hypoxia leading to endometrial sloughing. Although no specific histological changes were seen in perivascular cells in this study, the results of detailed immunohistochemical studies are not yet available.

The thick-walled aggregates of vessels resemble those seen in the stroma of endometrial polyps, and it is likely that in asoprisnil-treated subjects these evolve from the thinner-walled aggregates as time goes on. The process appears specific to endometrium, as no similar changes were identified in the limited range of non-endometrial tissues examined in this study. In the endometrium, it is possible that there is an active process of angiogenesis stimulated by hypoxia through an action on the perivascular cells. However, the development of vascular aggregates may be analogous to the stromal vessels of endometrial polyps, where it is believed that focal areas of non-shedding endometrium persist for several cycles to form the polyp. In both situations, therefore, endometrium is not shed at menstruation, and this may be the mechanism whereby such morphologically abnormal vessels develop. However, although some similarities with stromal changes of endometrial polyps were observed, with asoprisnil, the endometrium remained flat and often thin, without gland crowding and without prominent stromal collagen. It is notable that there was a low incidence of polyp formation in the asoprisnil-treated patients in this study, with only one small polyp found in one subject taking 10 mg.

No histological changes were found in this 3-month study to give cause for concern about the development of malignant or premalignant changes. Glands were specifically assessed in each sample to identify features of cytological atypia, but none was found. The glandular architectural changes did not suggest complex hyperplasia, and although there was in some cases a superficial resemblance to simple cystic hyperplasia, this was merely due to the presence of dilated glands, whereas the other features of simple hyperplasia were lacking. Moreover, endometrial thickness was not increased; indeed, the trend (which was not statistically significant) was for a decreasing endometrial thickness in asoprisnil-treated patients. It is important that histopathologists are made aware of the potential diagnostic pitfalls of misdiagnosing endometrial polyps and simple hyperplasia, particularly in curettage or aspiration biopsies of endometrium from asoprisnil-treated patients.

There are some morphological similarities between the endometrial effects of asoprisnil in this study, and those of the PR antagonist mifepristone, but there are also significant differences. Cystic glandular dilatation with inactive epithelial appearances is common to both, and there is an antiproliferative effect (Baird *et al.*, 2003; Narvekar *et al.*, 2004). However, no specific vascular changes have been identified in the stroma in mifepristone-treated endometrium, and glands show a less tortuous morphology, generally showing simple tubular appearances or cystic dilatation. This suggests an absence of agonist activity and a pure antagonist effect with mifepristone on PR, whereas with asoprisnil, the gland tortuosity probably reflects a partial agonist effect in addition to antagonist action.

In this study, comparison of mitotic activity in endometrium between treatment and control groups is limited, as five out of seven subjects in the control group showed secretory phase appearances in which mitoses are not expected to be present. However, it is notable that the low level of mitotic activity in the asoprisnil treatment groups is in keeping with the results of studies of asoprisnil in primates (Brenner and Slayden, 2005), and may have parallels in studies of other PR ligands such as mifepristone, in which an antiproliferative effect has also been described (Baird *et al.*, 2003; Narvekar *et al.*, 2004).

Mitotic activity was also decreased in leiomyomata in patients receiving asoprisnil, and in keeping with this, degenerative changes were also more common. This is consistent with the observed diminution in leiomyoma volume in patients

ceiving asoprisnil (data not shown). However, no histological fferences were found in the non-leiomyomatous myometrium :tween placebo and asoprisnil groups. Asoprisnil does not em to induce any noticeable changes in the myometrium.

In conclusion, this study has shown that asoprisnil adminis-red as 10 or 25 mg daily doses for 3 months leads to the :velopment of unique histological appearances in the endo-etrium. As well as consistent effects on gland morphology, ick-walled muscular vessels are seen in the stroma which pear to be specific to endometrial tissue. The mechanism uterine bleeding suppression is still unknown, but it may volve interaction between asoprisnil, perivascular cells and iral vessels. This study reports, for the first time, a detailed aluation of endometrial changes induced with asoprisnil ter a 3-month treatment, based on the full-thickness endo-etrial samples obtained during hysterectomy. Histopatholo-sts examining endometrial biopsy specimens from oprisnil-treated patients need to be aware of the unique PRM effects' induced by asoprisnil (and perhaps other com-unds of this class) to avoid misclassifying appearances as mple hyperplasia or polyps, which share some of the features it have significant differences.

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onflict of interest

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Menorrhagia, mechanisms and targeted therapies

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Purpose of review

Efficient treatment options for the increasing complaint of heavy menstrual bleeding are required to avoid the morbidity associated with major surgery. This review summarizes recent developments in the nonsurgical approach to treating menorrhagia.

Recent findings

Even though the mechanism of menstruation has still not been fully elucidated, there have been promising developments in the attempt to reduce menstrual blood loss. The levonorgestrel-releasing intrauterine system has proven very efficient, and there is a frameless device suitable for enlarged uterine cavities. The side effect of breakthrough bleeding has been the main reason for discontinuing treatment. Recent studies have investigated options of concomitant therapy with antiprogestogens to counter this effect. Another group of novel compounds are selective progesterone receptor modulators, which thus far have been shown to reduce menstrual bleeding without the unwanted effect of unscheduled bleeding episodes.

Summary

As menorrhagia is largely a subjective complaint, it is important for successful therapy to be based on an informed patient's choice. Currently, lack of evidence-based practice, poor compliance and unpleasant side effects limit the success of medical therapies contributing to the amount of major surgery performed for this indication. If concomitant therapies succeeded in minimizing the episodes of breakthrough bleeding with the levonorgestrel-releasing intrauterine system, its recommendation as a first choice treatment may be confounded. Further research is needed into efficiency and safety of selective progesterone receptor modulators, which appear to have great potential in reducing menstrual blood loss.

Keywords

heavy menstrual bleeding, informed choice, levonorgestrel-releasing intrauterine system, medical therapy, selective progesterone receptor modulators

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Abbreviations

LNG-IUS	levonorgestrel-releasing intrauterine system
MMP	matrix metalloproteinase
NSAID	nonsteroidal antiinflammatory drug
SPRM	selective progesterone receptor modulator
VEGF	vascular endothelial growth factor
vWD	von Willebrand's disease

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Introduction

Menorrhagia is better described as heavy menstrual bleeding and has classically been defined as blood loss greater than 80 ml per month [1]. Even though it is rather impractical to actually measure the blood loss, it has recently been shown that the subjective estimate of the blood loss correlates with the measured loss better than previously believed [2]. However, it can be questioned how useful this criterion of 80 ml is in clinical practice as management is largely guided by the subjective complaint of heavy menstrual bleeding rather than measured figures [3].

According to currently proposed definitions, menorrhagia as a symptom refers to heavy menstrual bleeding irrespective of aetiology and irrespective of the regularity of the menstrual cycle. Dysfunctional uterine bleeding is a diagnosis given to patients with excessive uterine bleeding after any demonstrable pathology has been excluded and also comprises both anovulatory and ovulatory heavy bleeding [4].

Menstrual problems are among the most common reasons for specialist referral and account for a third of gynaecological outpatient workload. The complaint of menorrhagia is of increasing prevalence. Earlier onset of menarche and increased life expectancy as well as the ability to regulate fertility and less time spent breastfeeding all contribute to the fact that women experience more periods in their lifetime than their predecessors a century ago [4,5]. Due to more demanding lifestyles of working women, there is probably also a reduced tolerance of troublesome periods and a trend to seek professional advice.

As menorrhagia is largely a subjective complaint, the main aim of treatment has to be improved quality of life. Expectations of treatment and the treatment options offered are influenced by psychosocial and cultural factors, as reflected in the wide variation in hysterectomies

performed for menstrual disturbance [6]. Hysterectomy rates vary significantly with the patient's educational background [7] and also with the extent to which women are involved in the process of decision-making regarding the appropriate treatment [8].

Considering the cost and morbidity of a hysterectomy, it is highly desirable to develop alternative treatment options for menorrhagia. The trend of women preferably avoiding major surgery when adequately counselled [8] and the need for fertility-conserving treatment in an increasing number of women choosing to postpone childbearing to a later age can only add to the urgency of this quest. The aim of this review is therefore to give an overview of current knowledge and ongoing research into medical therapies for menorrhagia.

Causes of heavy menstrual bleeding

To date, no clear mechanism has been identified that would explain the symptom of heavy menstrual bleeding in women with no other pelvic pathology or systemic disorder. No correlation has been established between endometrial histology and objectively measured blood loss. Neither has there been any evidence of gross ovarian or hypothalamic–pituitary dysfunction in women complaining of heavy menses [5]. At present, the search for targeted therapies will therefore have to focus on the mechanism of menstruation itself in order to try and modulate this physiological process.

Mechanism of menstruation

In the presence of ovulatory cycles, withdrawal of progesterone triggers a cascade of molecular and cellular events within the endometrium, initiating its breakdown and culminating in menstruation. Studies into the local endometrial response to progesterone withdrawal suggest that key inflammatory mediators such as interleukin-8 (IL-8), monocyte chemotactic peptide-1 and cyclooxygenase 2 are upregulated [9]. A recent study proposes that the pathway to stimulate cyclooxygenase 2 mRNA expression in endometrial stromal cells following progesterone withdrawal is via decreased copper–zinc superoxide dismutase activity and consequent increase in reactive oxygen species [10]. Simultaneous inhibition of prostaglandin dehydrogenase results in an elevation of prostaglandin concentration (PGE and PGF2α). With a subsequent influx of leukocytes, menstruation resembles an inflammatory event [11].

It has been recognized that there is an increased synthesis of PGE_2 and PGE-binding sites in the tissue of women with menorrhagia. Recent studies into the function of prostaglandin receptors in endometrium have shown that prostanoids promote angiogenesis and may have a role in aberrant neovascularization leading to dysfunctional uterine bleeding. In particular, the *trans*-phosphorylation of receptor tyrosine kinases by prostanoid receptors may be crucial in this process and may be amenable to modulation by drug treatment. It has been postulated that a prostanoid receptor antagonist with a receptor tyrosine kinase inhibitor may be a promising combination in the search for an effective therapy of menstrual disturbances [12•].

It has been fully appreciated that menstruation is a multifactorial event which can be divided into two phases. The first phase is hormone dependent and reversible with add back of progesterone within 36 h. It involves vasoconstriction of the endometrial spiral arteries and cytokine changes. The latter phase is not reversible by progesterone and includes activation of lytic mechanisms which are then inevitable [13]. Activation of matrix metalloproteinases (MMPs) such as MMP-3 with the potential to degrade the extracellular matrix can lead to a whole cascade of subsequent activations of lytic enzymes and eventually to tissue sloughing and menstrual bleeding [14,15]. So far very little is known about the mechanism of the subsequent endometrial repair, even though it is likely to be of equal importance for the understanding of abnormal uterine bleeding [15].

Other factors have been shown to contribute to the menstrual process such as endothelin with potent vasoconstrictor and growth factor properties. The expression of endothelin and its metabolizing enzyme neutral endopeptidase varies across the menstrual cycle and differences in endothelin immunoreactivity have been identified in the endometrium of women with excessive menstrual bleeding versus women with normal menstruation. It has been postulated that the finding of reduced endothelin in the endometrium of women with excessive blood loss is associated with an impairment of postmenstrual vasoconstriction to cease the bleeding and impairment of endometrial regeneration and repair [16,17].

The role and timing of hypoxia in the whole process is the subject of current research. It has been acknowledged that vascular endothelial growth factor (VEGF), a prominent angiogenic factor, is stimulated by hypoxia and plays an important role in the menstrual process [9]. Both hypoxia and VEGF may well be involved in the aetiology of abnormal bleeding, and a further understanding of the exact mechanisms will facilitate the development of effective drug therapies.

Uterine pathology

Structural abnormalities of the uterus such as endometrial polyps, adenomyosis and leiomyomata are well known to be causative factors of excessive uterine bleeding. Detection of such pathology and ruling out the presence

of malignancy are the main reasons for investigations in a woman presenting with heavy menstrual blood loss.

Leiomyomata, or uterine fibroids, are the most common benign pelvic tumours of the female genital tract, affecting approximately 20–25% of all women of reproductive age [18]. However, not all uterine fibroids are symptomatic. A recent study showed no correlation between menstrual blood loss and number, size and location of the fibroids. Only submucous fibroids were consistently associated with heavy menstrual blood loss. It was also found that the majority of fibroids except those in a subserosal location do distort the uterine cavity which possibly accounts for their potential to cause symptoms [18].

Due to the inconsistent association between uterine fibroids and heavy uterine bleeding and the fact that the report of menstrual disturbances is influenced by psychosocial factors [6], a study was undertaken to investigate whether women report different symptoms after being given a diagnosis of uterine fibroids. However, there was no evidence that symptom reporting was influenced by the knowledge of the diagnosis [19].

It is still largely unknown how exactly leiomyomata cause abnormal uterine bleeding. Abnormalities in the vasculature of leiomyomatous uteri were described as far back as 1912 [20]. More recently, the molecular mechanisms of these vascular abnormalities have been investigated. Several vasoactive growth factors such as basic fibroblast growth factor, VEGF, heparin-binding epidermal growth factor, platelet-derived growth factor, transforming growth factor-β, parathyroid hormone-related protein and prolactin are differentially regulated in leiomyomata and could therefore potentially act as mediators of leiomyoma-related excessive uterine bleeding. Postulating that dysregulated angiogenesis results in abnormal vessels, which contribute to the symptom of heavy bleeding, these angiogenic growth factors could be a potential target for novel therapies. Angiogenesis as a physiological process is unique to the female reproductive system. Usually it is a pathological process occurring in wound healing and tumour formation. Therefore, modulating agents are likely to have minimal side effects and could have great potential in the treatment of leiomyoma-related complications [21].

Systemic disorders

Heavy menstrual bleeding may be associated with systemic disorders such as thyroid, liver or renal disease. To be considered foremost are coagulation disorders such as von Willebrand's disease (vWD) and platelet defects. There is an ongoing debate as to whether bleeding disorders should be screened for in women complaining of excessive menstrual blood loss. The Royal College of Obstetricians and Gynaecologists [22] states in its guideline for the management of menorrhagia in secondary care that tests for bleeding disorders should only be performed in the presence of other suggestive symptoms or findings on examination. The evidence supporting screening for vWD in particular has recently been reviewed again. vWD was confirmed in 5–20% of women presenting with heavy menstrual blood loss but there were insufficient data to justify routine testing [23]. Other studies have found similar [24,25] or even higher figures [26,27], and another review concluded that testing for vWD should be considered in the absence of any other pathology [25]. Particular awareness seems prudent when adolescents present with heavy menstruation [28]. To avoid the use of the expensive laboratory-workup to confirm vWD as a screening tool, a simpler platelet function analyser (PFA-100) has recently been demonstrated to be both sensitive and specific in the detection of vWD and other bleeding disorders [29].

Management of heavy menstrual bleeding

Management of heavy menstrual bleeding can generally be divided into medical and surgical therapy. Currently available options have recently been summarized emphasizing the importance of women making their own informed choices about their treatment [30•].

Surgical procedures, which conserve the uterus and are therefore less invasive than a hysterectomy, have been introduced within the last two decades and are still the subject of ongoing research. Endometrial resection or ablation and uterine artery embolization especially in the presence of uterine fibroids have all been shown to improve the symptom of heavy menstrual bleeding considerably [31–35]. Compared with a hysterectomy, these procedures carry a substantially lower risk of morbidity [36–38]. However, they are still suboptimal options for women wishing to retain their fertility. Pregnancy rates following endometrial ablation are below 1% [39] and associated with a risk of significant morbidity [40]. The outcome of pregnancies following uterine artery embolization appears more promising [41] but experience is still limited. For women who have not completed their families, drug treatment is therefore the only option and this review concentrates on medical therapy of heavy menstrual bleeding.

Medical therapy

The search for the ideal medical therapy, which is effective, affordable and acceptable, is ongoing. Drug therapy is the least invasive and expensive option for managing heavy menstrual bleeding and should be the initial treatment of choice. Lack of evidence-based practice, poor compliance and unpleasant side effects

have limited the satisfaction rates associated with medical therapy [42••]. In order to make drug therapy successful, it is as important to individualize the approach to management and involve the patient in this process as it is to develop new pharmaceutical agents.

This review focuses on recent studies and developments regarding the drug treatment of heavy menstrual bleeding. It is beyond the scope of this review to comment on all the currently established treatment options, which have recently been presented in a comprehensive review [42••].

Nonsteroidal antiinflammatory agents and antifibrinolytics

There is mounting evidence supporting the usefulness of nonsteroidal antiinflammatory drugs (NSAIDs) which already have an established place in the treatment of menstrual disturbances [12•]. More specific cyclooxygenase 2 inhibitors are not yet widely available [43] but may have an improved side-effect profile and could be more acceptable than commonly used NSAIDs.

The most commonly prescribed antifibrinolytic agent is the plasminogen activator inhibitor tranexamic acid. Even though most studies have demonstrated a favourable side-effect profile with no increased incidence of thrombogenic events [42••,44], it seems prudent to bear in mind the potential to cause adverse effects on the cardiovascular system. A recent case report on a possible relationship between acute myocardial infarction and the concomitant use of tranexamic acid and the combined oral contraceptive pill calls for further evaluation of this potential association [45].

Progestogens

Progestogens have been used to control menstrual disturbances by various routes of administration. Cyclical administration of oral progestogens such as norethisterone, even though widely used, is ineffective in women with ovulatory cycles unless prescribed for 21 days from day 5 of the menstrual cycle [46]. The parenterally administered long-acting contraceptive agent medroxy progesterone acetate and implants commonly reduce menstrual blood loss. However, due to a high rate of adverse effects and concerns regarding the long-term effects on bone density [47], they are a less favourable option for treatment of menorrhagia in women not requiring contraception [42••].

The use of oral danazol, an isooxazole derivative of 17α-ethinyltestosterone, has also been limited by its side-effect profile. A recent study on the efficacy and safety of vaginal danazol confirmed the effect of reduced menstrual blood loss and regression of endometrial hyperplasia but also a high rate of discontinuance with the treatment [48].

The levonorgestrel-releasing intrauterine system (LNG-IUS, Mirena) has become the most favourable route of administering exogenous progestogens. It has consistently been shown to reduce menstrual blood loss with amenorrhoea rates up to 33% by causing endometrial atrophy [49]. This has led to the recommendation to use the LNG-IUS as a first-line option to treat menorrhagia [50].

Recent randomized trials have assessed the efficiency of the LNG-IUS compared with endometrial resection and hysterectomy. The LNG-IUS was found to be comparable to endometrial resection in its efficiency in a randomized trial with 3-year follow-up [51•]. It has been shown before that even though in the short term surgery may be more successful [52], the LNG-IUS is as effective as endometrial resection after the first year [53]. A randomized controlled trial with 5-year follow-up comparing the outcomes of the LNG-IUS insertion with hysterectomy found no difference in quality of life improvement but a significant difference in cost effectiveness [54••]. The cost of treatment with LNG-IUS was half of the cost of hysterectomy after 5 years. The conclusion that the LNG-IUS is the most effective medical therapy for menorrhagia, avoiding major surgery in 60% of patients, consolidates the recommendation that it should be a first-line treatment [55]. The LNG-IUS has also been found to be effective and well tolerated in women with inherited bleeding disorders in whom treatment of menorrhagia had previously been unsuccessful [56].

A more recent development is a 'low-dose' FibroPlant-LNG-IUS delivering 14 μg/day. This frameless, anchored device has been shown to have equally profound effects on menstrual blood loss and ferritin levels. Its design accounts for minimized occurrence of pain and expulsion [57•] and also allows its use in enlarged or distorted uterine cavities [58].

The most frequent indication for discontinuance of treatment with progestogens including the LNG-IUS is unscheduled breakthrough bleeding. Despite extensive research, as yet no single mechanism has been identified to explain this phenomenon. Various effects of prolonged exposure to progestogens on endometrial morphology, sex steroid receptor expression, expression of inflammatory mediators and endometrial vasculature have been studied. Features of leukocyte infiltration and aberrant angiogenesis leading to increased vascular fragility have recurrently been described [59–61,62•,63–65].

Studies comparing tissue from long-term progestogen users complaining of unscheduled bleeding with asymptomatic users in particular have also reported changes in histological appearance and levels of inflammatory and

angiogenic factors in symptomatic patients. A recent study showed that the endometrium from users with regular cycles predominantly exhibited a progestogen effect whereas those with irregular bleeding often demonstrated proliferative endometrium. There were also signs of stromal breakdown and vascular thrombi in the tissue from patients with breakthrough bleeding as well as significantly more intense immunostaining of IL-13 and IL-15 [66]. Others have shown an increased number of leukocytes, increased MMP-3 [67] and an increased level of serum nitric oxide metabolites in women with irregular bleeding [68]. All these findings confound the hypothesis that inflammation and dysregulated angiogenesis are the major contributors causing this undesirable symptom. However, so far, treatment of irregular bleeding associated with use of long-term progestogens is still largely empiric, consisting of sex steroids and NSAIDs [66].

It has been suggested that aberrant angiogenesis may be caused by a reduced local endometrial ratio of angiopoietins Ang-1 : Ang-2. This may be altered by hypoxia/reperfusion injury and subsequent production of free radicals due to reduced endometrial perfusion which has been reported in progestogen users [69]. Ang-1 and Ang-2 have complimentary roles in regulating angiogenesis. Both act on the same endothelial receptor Tie-2. Whilst Ang-1 activates the Tie-2 receptor and is important for maintaining vascular integrity, Ang-2 has an antagonistic effect and enhances vascular permeability [65]. A reduction in the endometrial Ang-1 : Ang-2 ratio by free radicals would implicate a potential role for antioxidant therapy, such as vitamin E supplementation. The activation of stress-activated kinases resulting from hypoxia/reperfusion injury could be blocked by oxygen radical scavengers *in vitro* [69].

Based on the assumption that changes in the endometrial vasculature do not persist when the endometrium becomes atrophic as symptoms improve with continued use of progestogen, it has been considered that pretreatment regimens or concomitant agents to render the endometrium atrophic may prevent unscheduled bleeding. Antiprogestogens or inhibitors of MMP activity have been suggested [59,70]. There are data supporting the use of the antiprogestogen mifepristone administered once monthly in combination with the levonorgestrel-releasing subdermal implant to improve the bleeding pattern [59,71,72]. The antiprogestogens ORG 31710 and ORG 33628 have also been used in combination with the progestogen-only pill desogestrel and were found to reduce vaginal bleeding. Administering ORG 31710 once monthly to women using desogestrel resulted in an improved cycle pattern, albeit the effect was only temporary [73,74••]. In another trial ORG 33628 was given in a continuous combined regime with a daily dose in addition to desogestrel and effected a dose-dependent reduction in bleeding episodes. However, if progestogens are used for contraception rather than treatment of excessive bleeding, consideration has to be given to the compromised inhibition of ovulation that may occur with the concomitant use of an antiprogestogen [74••].

Selective progesterone receptor modulators

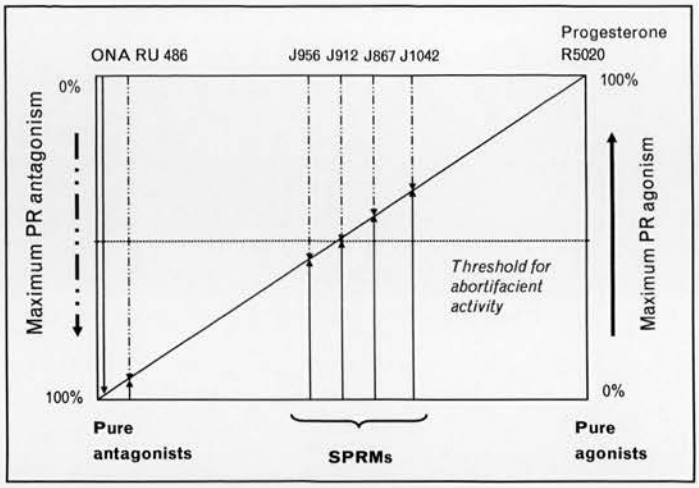
Subsequent to the original description of the antiprogestin mifepristone, numerous related progesterone receptor ligands with mixed agonist and antagonist properties have been developed and studied in animal models and various clinical settings [75]. Different compounds have varying degrees of progesterone agonistic and antagonistic properties (Fig. 1) [76]. It is notable that in contrast to progesterone antagonists, selective progesterone receptor modulators (SPRMs) have only slight labour-inducing activity [75]. It has been established that progesterone antagonists as well as SPRMs with partial agonist activity reduce menstrual blood loss [77]. This antiproliferative effect on the endometrium was unexpected as SPRMs do not suppress ovulation and lead to increased endometrial expression of estrogen and progesterone receptors. The mechanism of suppressed endometrial growth has not been fully elucidated. There seems to be a direct effect of SPRMs on the endometrial vasculature. Increased expression of the androgen receptor may also be a contributing factor [75,78].

The use of mifepristone for the treatment of uterine leiomyomata and the associated symptom of heavy menstrual bleeding has recently been reviewed, confirming a reduction in blood loss with amenorrhoea rates between 63 and 100%. The review included 166 women who had received 5–50 mg mifepristone for 3–6 months. Transient elevation of transaminases was noted in 4% and endometrial hyperplasia was found in 28% of patients screened [79•]. However, studies using lower doses of between 2 and 5 mg mifepristone showed an absence of proliferative activity in the endometrium and even a significant decrease in the expression of the mitosis marker phosphorylated histone H3 compared with controls [80•,81].

The novel SPRM asoprisnil (J867) has also been shown to cause a dose-dependent suppression of menstruation [82]. Its effect has been studied in healthy premenopausal women with regular menstrual cycles [83••] and in patients with symptomatic uterine fibroids [84••]. So far, asoprisnil has consistently caused dose-dependent suppression of endometrial proliferation with consequent reversible amenorrhoea in the absence of oestrogen deprivation. The side effect of unscheduled breakthrough bleeding which has been limiting the usefulness of long-term progestogens has thus far not been reported

Figure 1. Properties of selective progesterone receptor modulators (SPRMs)

Spectrum of antagonistic and agonistic activities of progesterone receptor (PR) ligands based on studies in in-vivo animal models (guinea pigs, rabbits). ONA, onapristone; R5020, pure progestin promegestone; J867, asoprisnil. Source: Reproduced with permission from Schubert *et al.* [76].



with asoprisnil. Overall, the compound has been well tolerated and had a favourable safety profile [83••,84••]. Further studies are ongoing but the potential of this compound for the treatment of heavy menstrual bleeding and other symptoms associated with uterine fibroids appears promising.

Conclusion

Advances in the development of medical therapies for heavy menstrual bleeding are highly desirable to reduce costs of treatment and provide options for women wishing to retain their fertility.

Studies into the mechanism of menstruation have pointed out potential targets for modulating pharmaceutical agents. At present, options of medical therapy mainly comprise NSAIDs, antifibrinolytics and progestogens. Whilst the LNG-IUS has proven very effective in reducing menstrual blood loss, the usefulness of exogenous progestogens has been limited by the side effect of unscheduled breakthrough bleeding. The idea of concomitant administration of antiprogestogens to improve the cycle pattern appears promising but warrants further research.

Whilst in a majority of women suffering from heavy menstrual bleeding no pathology can be demonstrated, uterine fibroids are a common causative factor for this symptom. Studies into the molecular mechanism of fibroid-related excessive uterine bleeding have identified a variety of angiogenic growth factors as potential targets for drug therapy with possibly minimal side effects. Thus far, this approach has not been pursued any further.

The antiproliferative effect on the endometrium of the most promising group of compounds, the SPRMs, was initially unexpected. The mechanism of suppressing the endometrial growth is still unclear but appears to be primarily due to a direct effect on the endometrial vasculature. So far, the SPRM asoprisnil used in clinical trials studying women with and without uterine fibroids has consistently led to reversible amenorrhoea without any breakthrough bleeding. Results of further clinical studies are awaited with interest.

Whilst the search for new pharmaceutical agents to modulate menstrual bleeding is ongoing, it must be remembered that adequate counselling and involving the patient in the decision about her treatment is as important for the success of her therapy as the properties of any compound.

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